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Including immunotoxicity in water quality assessment

Colophon

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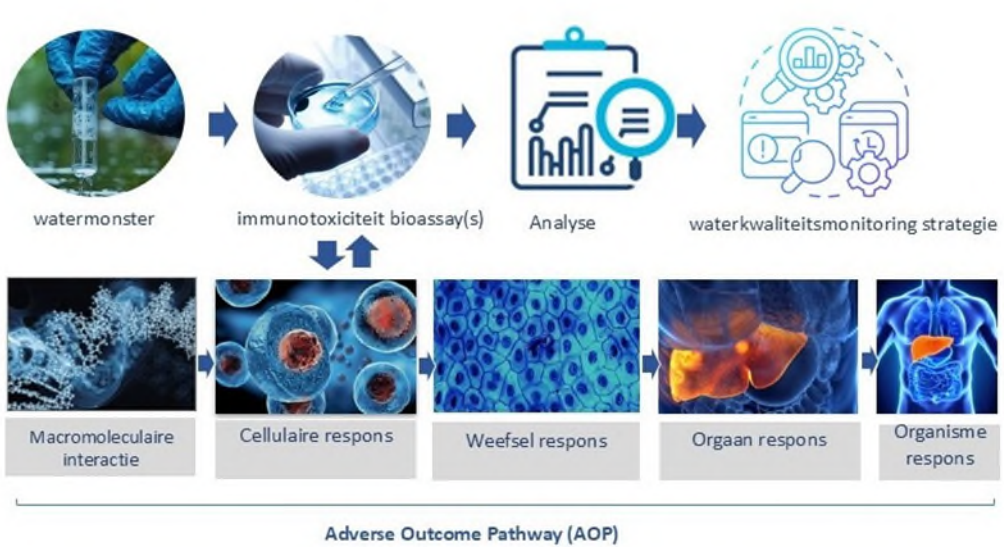
Managementsamenvatting

Redenen en routes om immunotoxiciteit op te nemen in de waterkwaliteitsbeoordeling

Auteurs: Sanah Majid, Daniel Duarte, Tessa Pronk

Met de opkomst van verontreinigingen zoals PFAS en Bisfenol A (BPA), waarvan bekend is dat ze de werking van het immuunsysteem verstoren wordt het steeds noodzakelijker om immunotoxiciteit mee te nemen bij de beoordeling van waterkwaliteit. Op basis van literatuuronderzoek, databaseonderzoek en gesprekken met experts is de huidige kennis over immunotoxicologie samengevat, zijn stoffen met immunotoxisch potentieel geïdentificeerd en is er een overzicht gemaakt van de beschikbare geavanceerde testmethoden. De resultaten van dit onderzoek tonen het belang van immunotoxiciteit als een relevant eindpunt voor de beoordeling van chemische risico's in drinkwater en laten bovendien zien dat het belangrijk is bioassays te ontwikkelen en implementeren die specifiek immunotoxische effecten van waterverontreinigingen kunnen detecteren. “Adverse Outcome Pathways” (AOP's) worden voorgesteld als een ethische en vooruitstrevende benadering van chemische risicobeoordeling. Het rapport over dit onderzoek sluit af met praktische aanbevelingen voor waterbedrijven waarmee zij prioriteit kunnen geven aan het monitoren van (vermoedelijk) immunotoxische stoffen en toepassing van geavanceerde testmethoden om veilig drinkwater te garanderen, met het oog op opkomende verontreinigingen.



Illustratie van het testen op immunotoxiciteit voor drinkwaterkwaliteit: Beginnend vanaf links, het verzamelen van watermonsters, gevolgd door immunotoxiciteitstesten met behulp van geschikte bioassays. De resultaten worden vervolgens geanalyseerd en op basis daarvan wordt een bewakingsstrategie gekozen. De AOP-benadering kan een leidraad zijn bij de keuze van de geschikte bioassay en kan ook inzicht verschaffen in potentiële toxiciteitsmechanismen.

Belang: Immunotoxiciteitsbeoordeling integreren in waterkwaliteitsmonitoring en risicobeoordeling

Er is toenemend bewijs dat nieuwe verontreinigende stoffen zoals PFAS en BPA verband houden met verstoring van het immuunsysteem. Deze chemische stoffen zijn op grote schaal aanwezig in het milieu. Ze vormen daarmee een aanzienlijk risico voor de volksgezondheid, vooral bij kwetsbare bevolkingsgroepen. De traditionele kaders voor waterkwaliteitsmonitoring richten zich echter over het algemeen op meer gevestigde toxicologische eindpunten zoals carcinogeniteit. Immunotoxiciteits-testen zijn niet opgenomen in de routinebeoordelingen. Daardoor blijven potentiële

risico's in drinkwater onopgemerkt. Voor waterbedrijven is het belangrijk om te kunnen testen op risico's met betrekking tot alle relevante gezondheidseffecten.

Aanpak: Literatuuroverzicht, databaseanalyse en gesprekken met experts

Via literatuuronderzoek zijn relevante studies en ontwikkelingen op het gebied van immunotoxiciteitsbeoordeling op een rij gezet en immunotoxische stoffen geïdentificeerd die relevant kunnen zijn voor drinkwater. De verzamelde informatie kwam uit wetenschappelijke artikelen, reviews en databases rond regelgeving en milieutoxiciteit, waaronder het USEPA Integrated Risk Information System (IRIS) en ECOTOX. Ook werd de Nederlandse REWAB database geraadpleegd om informatie te verzamelen over de aanwezigheid van bekende immunotoxische stoffen in drinkwaterbronnen. Rapporten en richtlijnen van gerenommeerde organisaties, zoals het Rijksinstituut voor Volksgezondheid en Milieu (RIVM) en de Wereldgezondheidsorganisatie (WHO) werden ook bekeken om inzicht te krijgen in de huidige normen, methodologieën en richtlijnen voor immunotoxiciteitsbeoordeling. Bovendien werd input gevraagd van immunotoxicologie-experts van de Universiteit Utrecht en het RIVM om de bevindingen aan te vullen.

Resultaten: Inzicht in potentiële immunotoxische verontreinigingen en bruikbare testmethoden

Dit rapport geeft een wetenschappelijke basis voor het opnemen van immunotoxiciteitstesten in de waterkwaliteitsmonitoring en biedt een manier om immunotoxische risico's van bekende en opkomende verontreinigingen te evalueren om drinkwater beter te beschermen. Het beschrijft verschillende milieuverontreinigingen, waaronder pesticiden, farmaceutica en industriële stoffen, met bekende of potentiële immunotoxische effecten zoals immunosuppressie of -stimulatie, en biedt een overzicht van de momenteel beschikbare *in vivo*, *in vitro* en *in silico* immunotoxiciteits-testmethoden. Om immunotoxische risico's te voorspellen blijken 'Adverse Outcome Pathways' of AOP's waardevol te zijn. AOP's beschrijven de keten van causaal gekoppelde gebeurtenissen op verschillende biologische niveaus die kunnen leiden tot een schadelijk gezondheids- of ecotoxicologisch effect na een initiële moleculaire interactie. AOP's zijn ontwikkeld als hulpmiddel, ze vormen het centrale element voor een kenniskader dat helpt om chemische risico's te beoordelen op basis van een mechanistisch model. Ook benadrukken onze resultaten de meerwaarde van de inzet van 'New approach methods' (NAM): methoden die testen op toxiciteit zonder dierproeven. Een voorbeeld van NAM's zijn *in-vitro* bioassays die specifiek ontworpen zijn om toxische effecten in water te detecteren, zoals effecten die gerelateerd zijn aan immunotoxiciteit. Kennis over AOP's kan waterbedrijven helpen om de meest relevante bioassays te selecteren voor het detecteren van immunotoxiciteit.

Toepassing: AOP-gestuurde immunotoxiciteits-bioassays gebruiken in waterkwaliteitsmonitoring

Waterbedrijven worden aangemoedigd om immunotoxische stoffen op te nemen in hun risico-gebaseerde monitoringprogramma's en om innovatieve testmethodes zonder proefdieren te gebruiken om de betrouwbaarheid en volledigheid van risico-evaluaties te verbeteren. Zo kunnen drinkwaterbedrijven proactief nieuwe verontreinigingen aanpakken en zorgen voor een voortdurende bescherming van de volksgezondheid en naleving van de zich ontwikkelende regelgeving.

Rapport

Dit onderzoek is beschreven in het Waterwijs-rapport "Including immunotoxicity in water quality assessment" (BTO 2024.100).

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General Introduction

With an expanding list of regulatory parameters, the drinking water sector faces ongoing challenges to safeguard public health against a growing number of contaminants. Recent amendments to the EU Drinking Water Directive, such as the inclusion of Per- and Polyfluoroalkyl Substances (PFAS) and Bisphenol A (BPA) in national drinking water standards, reflect rising concerns about the health impacts of these anthropogenic contaminants (see: wetten.nl - Regeling - Drinkwaterregeling - BWBR0030152 (overheid.nl)**.** Studies show that exposure to micropollutants like PFAS and BPA can disrupt immune function, increasing susceptibility to infections and other health risks (Bolt et al., 2019; EFSA, 2020; US EPA, 2022a). Given this evidence, immunotoxicity is now recognized as a critical endpoint in assessing chemical risks in water sources. Emerging immunotoxic substances not yet covered by current European legislation could pose unforeseen health risks, emphasizing the need for new insights that would enable proactive measures in water quality management. Although the EU Drinking Water Directive currently lack a direct focus on immunotoxicity, it does integrate WHO health-based guidance values for selected substances based in part on immunotoxic effects.

Given that drinking water sources constantly face the threat of contamination by both known and emerging chemicals, understanding developmental and adult immunotoxic potential of (drinking) water-relevant chemicals is of particular importance for public health concerns. Exposure to immunotoxicants through (drinking) water can have widespread implications for individuals of all ages. Therefore, it is crucial to investigate chemical substances for their immunotoxic properties and assess the mechanisms through which these chemicals exert immunotoxic effects at different life stages. This knowledge will allow scientists, regulators and water utilities to work towards ensuring the safety of drinking water supplies to minimize potential adverse health outcomes associated with immunotoxicity by developing preventive measures to safeguard public health. The present project consolidates current knowledge on immunotoxicology, and provides an overview of known immunotoxic contaminants, and testing options to assesses their implications for water utilities. The output of this report can be used as a basis for future research to support current monitoring measures (within and beyond regulatory requirements).

The report is structured in six chapters. The first chapter introduces immunotoxicity and outlines the basic concepts of the human immune system to provide the basic knowledge for subsequent sections. The method used for obtaining information is provided in the second chapter. The third chapter includes the current methodologies for assessing immunotoxicity risks in drinking water, with an emphasis on predictive testing strategies and adverse outcome pathway (AOP) frameworks. This section explores how these frameworks can be applied to understand the links between contaminants and immune effects, including their potential use in effect-based monitoring. In the fourth chapter, information on the available immunotoxicity testing methods for hazard assessment in food and drinking water is provided. In the fifth chapter, a list of compounds with their immunotoxic potential has been drawn up. Data was filtered from US Environmental Protection Agency (US EPA) Integrated Risk Information System (IRIS) and US EPA ECOTOX databases by immunotoxic endpoint. To identify the compounds most relevant for water quality, this list was compared with the REWAB database (Dutch acronym for the registry of water utility water-quality data) including data on the presence of these compounds in drinking water sources. A final chapter (chapter 6) provides a discussion and conclusion, as well as recommendations for future research interests.

1 Introduction

1.1 Immune System

The immune system is a complex network of cells, tissues and organs that work together to support the body's defense mechanisms against foreign substances, while also eliminating damaged or abnormal body cells (Klenerman, 2017; Semwal et al., 2022) (see Figure 1). It is designed to be highly responsive, with both innate and adaptive components (in higher organisms) working together to protect the body from infections, tumors, and other harmful invaders. However, this responsiveness can sometimes become problematic, as inappropriate activation of immune system may result in conditions such as allergies, autoimmune diseases (AID), and prolonged inflammation (Furman et al., 2019; Miller et al., 2023; Wang et al., 2023). Allergies occur when the immune system overreacts to generally harmless substances, such as dust, pollen, or certain foods, triggering symptoms like rashes, swelling, or even life-threatening anaphylaxis (Wang et al., 2023). AID arise when the immune system attacks the body's own tissues, mistaking them for foreign invaders, with conditions such as lupus, rheumatoid arthritis, and type 1 diabetes among the most common examples (Miller et al., 2023). Prolonged inflammation, on the other hand, is a chronic immune response that persists beyond its beneficial purpose, often contributing to the development of diseases like cardiovascular disorders, cancer, and neurodegenerative conditions (Furman et al., 2019).

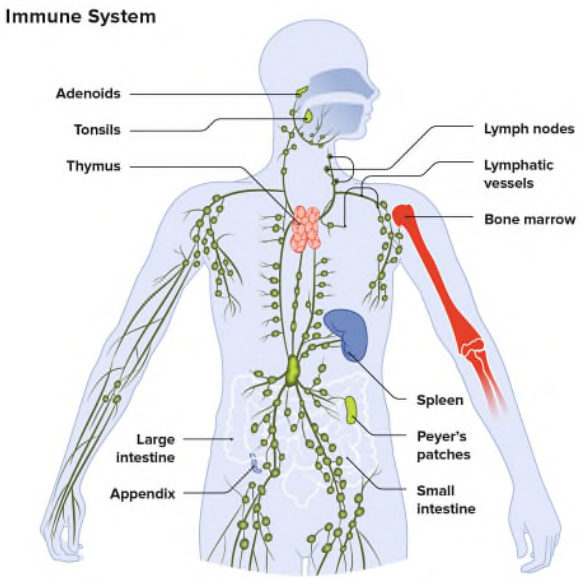

Figure 1. Human immune system. *The immune system consists of a range of components, including; primary lymphoid organs (bone marrow, thymus), secondary lymphoid organs (lymph nodes, spleen, mucosa-associated lymphoid tissue such as tonsils, Peyer's patch in small intestines, appendix), tertiary lymphoid tissues (liver, lungs) and barriers (epithelia) (image source: Medical news today)*

The immune system can be broadly categorised into two types: the innate immune system and the adaptive immune system (see Figure 2). Most cells participating in both innate or adaptive immune system originate from hematopoietic stem cells (HSCs), which reside primarily in the bone marrow in adults (and liver in fetus). These HSCs are multipotent stem cells meaning that they that have the capacity to differentiate into all types of blood cells, including those of the immune system (Descotes, 2004a; Khanlarkhani et al., 2016) (see Figure 3). The differentiation and development process of immune cells from HSCs is known as haematopoiesis. Haematopoiesis begins with a small population of self-renewing HSCs that give rise to two primary progenitor lineages: myeloid progenitor cells and lymphoid progenitor cells (Bou Zerdan et al., 2021). Each lineage leads to distinct types of immune cells, each with specialized roles in immune defense.

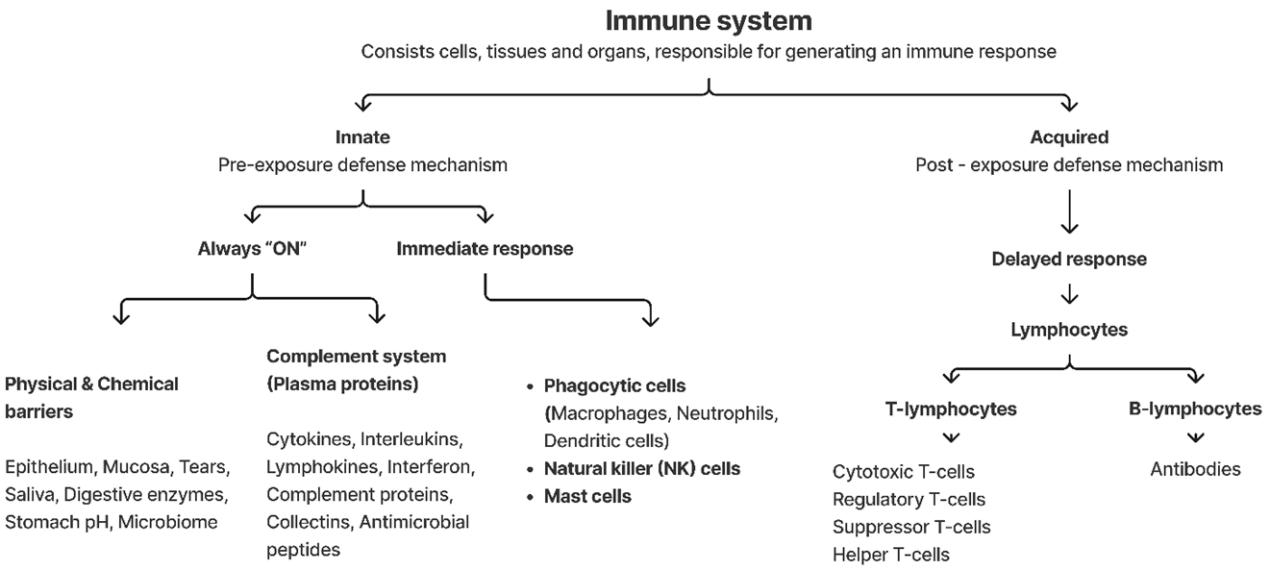

Figure 2: Overview of innate and acquired immune system *(Adapted from: Papenfuss & Bolon, 2014; Muller et al., 2019; Yu et al., 2023)*

The interaction between the innate and adaptive immune systems is essential for the body's robust defence against foreign substances, whether biological (e.g., pathogens) or chemical (e.g., pollutants) in nature. This complex system operates through tightly regulated, coordinated steps to prevent excessive immune activation or inadequate immune responses. The innate immune system serves as the body's first line of defence, providing immediate, non-specific defence mechanisms, (Weng, 2006; Nicholson, 2016; Strzelec et al., 2023). It comprises physical (e.g., epithelia) & chemical (e.g., saliva) barriers, as well as the macromolecular components such as plasma proteins (e.g., cytokines, interleukins) and cellular components such as, phagocytes (e.g., neutrophils, macrophages, dendritic cells), natural killer (NK) and mast cells. The cells of innate immune system respond rapidly to pathogens in a non-specific manner and help to activate the adaptive immune system (gate keepers of adaptive immunity).

The adaptive immune system, is a specialised and more targeted defence mechanism that develops over time in response to specific pathogens and comprises B lymphocytes (B-cells: bone marrow or bursa-derived cells) and T lymphocytes (T-cells: thymus cells) (Weng, 2006; Nicholson, 2016). B-cells are involved in humoral immunity, which is the immunity mediated by macromolecules. They produce antibodies that neutralize pathogens or foreign substances, such as toxins, and opsonize these targets, i.e., enhance their recognition and destruction by complement proteins and innate immune cells, such as macrophages and neutrophils. T-cells on the other hand are responsible for cell-mediated immunity , which does not rely on antibody production. Instead, these activate white-blood cells to destroy infected or cancerous cells (Weng, 2006; Nicholson, 2016; Strzelec et al., 2023).

Immunoregulatory cells, at multiple levels, including but not limited to regulatory T cells (Tregs), play a critical role in maintaining immune homeostasis and promoting immune tolerance (Cuturi & Anegon, 2010). These cells help prevent excessive immune activation, ensuring the immune system is appropriately responsive without leading to autoimmunity or chronic inflammation. Factors influencing this balance in the immune system range from signaling pathways[1] triggered by T-cell receptors, co-stimulatory receptors (cell surface molecules that enhance or modulate

[1] Signaling pathways are series of molecular interactions and biochemical reactions within a cell that control and coordinate cellular activities such as cell division or cell death (NCI Dictionary)

the activation signals), and cytokines, to various metabolic pathways, neuro-endocrine factors and the intestinal microbiota (Golub et al., 2010; Lee, 2018; Galbiati et al., 2021).

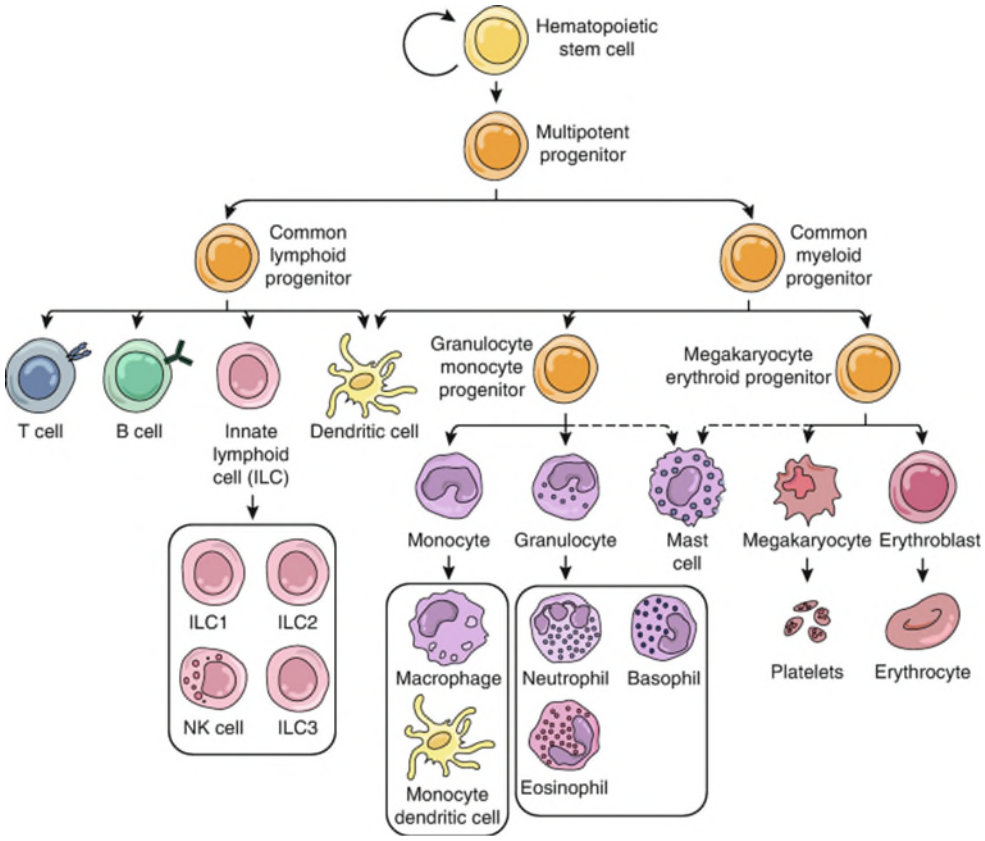

***Figure 3**. Origin of cells participating in innate or adaptive immune system (Image source: Kavathas et al., 2019)*

1.2 Immunotoxicity

Immunotoxicity refers to the adverse effects on the immune system resulting from the exposure to toxic substances (Gulati and Ray, 2009; Abdollahi and Behboudi, 2014). Disruptions to the immune system, whether through suppression or overactivation, can have significant health implications. These effects can arise via both direct and indirect pathways (Lankveld et al., 2010). Direct immunotoxicity occurs when a toxicant directly interacts with the immune system, leading to direct damage, and mainly includes immunosuppression (Lankveld et al., 2010; Wang et al., 2022). This suppression weakens the body's ability to defend against infections (host resistance) and may also increase the risk of certain non-genotoxic cancers, as the capacity of immune system to detect and eliminate abnormal cells is compromised (Lankveld et al., 2010). Indirect immunotoxicity refers to the adverse effects on the immune system that do not occur through direct interaction with immune system, but rather through the influence of other biological systems or pathways (Stølevik et al., 2011). The mechanisms of indirect immunotoxicity often involve complex pathways, where changes in neuro-endocrine-immune interactions alter the immune response (Galbiati et al., 2021). The interconnected nature of these three systems, referred to as neuroendocrine-immune network (NEI) are crucial for maintaining immune homeostasis and responding to various physiological challenges (Liu et al., 2016; Klein, 2021). The proper functioning of the immune system, therefore, depends on the regulation and balance of this complex network, where each system (immune, neuro, and endocrine) relies on the others for optimal operation (ThyagaRajan et al., 2012).

Clinically significant immunotoxic effects can be broadly classified into four main non-mutually exclusive types including immunosuppression, immunostimulation, hypersensitivity and autoimmunity (Germolec, 2010; Semwal et al., 2022). Immunosuppression and immunostimulation manifest as the inhibition or overactivation, respectively, of immune cells or their functions in the immune tissues. These responses are collectively termed as "direct immunotoxic effects" (Wang et al., 2023). Immunosuppression can arise from the depletion of immune effector cells or the disruption of intracellular pathways essential for antigen recognition and other elements of the immune response (Rice, 2019). Various environmental contaminants have been shown to significantly suppress or enhance immune responsiveness depending on exposure level, time and route of exposure (Kreitinger et al., 2016). A compromised or suppressed immune system may render the body more susceptible to infections or inflammatory responses. For example, persistent immunosuppression has been shown to present a risk of cancer (Rice, 2019).

Several studies have demonstrated higher vulnerability of the developing immune system, as well as unique or more persistent immune effects in children compared to that of adults (Luebke et al., 2006; Rowe et al., 2008; Holsapple & O'Lone, 2012; Wang et al., 2019; Mlelwa et al., 2024). This higher vulnerability is attributed to different factors, such as, unique immune maturational events that occur during critical windows of vulnerability early in life, making the developing immune system particularly susceptible (Dietert, 2008a). These critical windows are moments when the immune system is developing specific cells or organs, including the development of T-cells in the thymus and the establishment of immune repertoires. Disruption during these processes can lead to long-term immune dysfunction (Dietert, 2008a). The fetal origins of adult disease (FOAD) hypothesis suggests that chronic diseases seen in adulthood, including autoimmune diseases, result from prenatal exposure to environmental contaminants resulting in long-lasting or persistent effects, emphasizing the importance of understanding developmental immunotoxicity (Dietert et al., 2006; Singh et al., 2010; Karthikeyan et al., 2024). Within this context, endocrine-disrupting chemicals (EDCs) like BPA have for example emerged as significant contributors to developmental immunotoxicity (Hong et al., 2002; Hao et al., 2008; Vidal et al., 2021). However, the causality is not fully established and remains an active area of research with significant debate. Disruption in the development, functioning or coordination of immune system, such as caused by chemical substances (immunotoxicants), can lead to immunotoxicity, making the body vulnerable to dysfunction and increased susceptibility to diseases (Karol, 1998; Vinay et al., 2015; Lee 2018; Semwal et al., 2022).

1.3 Sources of Immunotoxicants

Immunotoxicants can originate from diverse sources and exposure to these substances may occur through ingestion, inhalation or skin contact (Bou Zerdan et al., 2021). Several immunotoxicants have been identified in drinking water sources globally, including PFAS, trichloroethylene (TCE), BPA and organotin compounds (EFSA, 2020; WHO, 2020; USEPA, 2022; Nguyen et al., 2023; Wee and Aris, 2023; Grunfeld et al., 2024). PFAS have been identified as immunotoxicants, with human exposures occurring globally (Grandjean et al., 2017; Grunfeld et al., 2024). Studies have associated certain PFAS to reduced vaccine antibody concentrations and can be immunotoxic in laboratory animals (Stein et al., 2016; EFSA, 2020; Grandjean et al., 2020; EFSA 2024). Similarly, BPA has been identified as a potential endocrine disruptor and immunotoxicant (Chen et al., 2018). Trichloroethylene (TCE) at high concentrations has been associated with irreversible effects on the gut microbial community and gut-associated immune responses (Khare et al., 2019). Furthermore, co-exposure to mercury has been found to increase the immunotoxicity of TCE, emphasizing the potential risks associated with mixtures of these substances in drinking water sources (Gilbert et al., 2010). Organotin compounds that may be present in source water or in the drinking water through leaching from PVC piping in the distribution system have also been evaluated for immunotoxic effects (Hendriksen et al., 2014; WHO, 2020). However, in the Netherlands, where copper lining is used in the piping, this does not present a relevant concern. It may be more applicable to areas with PVC pipes that could potentially leach these compounds into the water supply. Tetrabromobisphenol A (TBBPA), a flame retardant, has been identified as a immunotoxicant leaching into water from landfills (Feng et al., 2013; Park et al., 2014; Wang et al., 2020). This wide range of substances emphasizes the need to study the immunotoxicological potential of individual contaminants as well as mixtures in drinking water sources.

2 Method

We conducted a literature search to identify and evaluate relevant studies and advancements in the field of immunotoxicity assessment, as well as potential immunotoxic substances. Information was sourced from peer-reviewed articles, reviews, and meta-analyses. Additionally, we consulted regulatory and environmental databases such as IRIS, ECOTOX, and the Dutch REWAB database to gather information on (the presence of) known immunotoxic substances in drinking water sources. Reports and guidelines from reputable organizations, including, the Dutch National Institute for Public Health and the Environment (RIVM), the European Food Safety Authority (EFSA), the U.S. Environmental Protection Agency (USEPA), the Organisation for Economic Co-operation and Development (OECD) and the World Health Organization (WHO) were also reviewed to gain insights into current standards, methodologies and guidelines in immunotoxicity assessment. A combination of targeted keywords and phrases, such as "immunotoxicity assessment," "immunotoxic substances," "*in vitro* immunotoxicity testing," "in vivo immunotoxicity testing, " " immunotoxicity risk assessment," "immunotoxicants", "immune system disruption", "Immune system toxicity", "immune system dysfunction," "toxic effects on immune system" "*in vitro* immunotoxicity", *"in vivo* immunotoxicity", "immunoassays", "environmental immunotoxicants", "emerging contaminants", "wastewater immunotoxicity", "adverse outcome pathways for immunotoxicity", "next generation immunotoxicity testing", "immune biomarkers", "waterborne contaminants", "low-dose exposure and immune disruption', "regulatory immunotoxicity testing", and additional relevant variations were used to ensure coverage of the topic. The selection criteria included prioritization of studies focusing on immunotoxicity assessment methods or identifying substances with potential immunotoxic effects, with a preference for literature published within the last ten years. Only peer-reviewed articles and reports from reputable sources were included to ensure the reliability of the information. Furthermore, studies that employed methodologies, encompassing *in vitro* and *in vivo* approaches to assess immunotoxicity were prioritized. While we considered global studies, particular attention was given to research literature relevant to the Dutch context and European regulations.

3 Description of different types of immunotoxicity from 'key event' to 'adverse outcome'

Regulators face a dual challenge of evaluating an ever-growing number of chemicals while reducing reliance on animal testing. The Adverse Outcome Pathway (AOP) framework is a structured approach that provides a scientifically robust approach to predict chemical toxicity based on mechanistic insights (Villeneuve et al., 2014; McMinn et al., 2019). AOPs map the progression from a molecular initiating event (MIE), the initial interaction between a chemical and a biological target, through key events (KEs) to an adverse outcome (AO) of (regulatory) concern, such as disease (Cho et al., 2022). The KEs represent measurable biological changes at various levels of biological organization, including cellular, tissue, and organ levels (McMinn et al., 2019). A single MIE can lead to a cascade of downstream KEs, which can diverge and lead to various toxicological outcomes (Spinu et al., 2019). Alternatively, multiple MIEs can converge into a single adverse outcome (see Figure 4). Overlapping MIEs and KEs in AOP networks result in complex network patterns that help identify critical biological control points. In these complex networks, multiple AOPs converge towards a common node , and simultaneously diverge into multiple downstream effects with nodes acting as important control points. These structures, often referred to as bow-tie motifs, are essential for understanding how different pathways interact and influence toxicity (Kanpen et al., 2018). For example, the overlapping pathways reveal interactions between AOPs that may result in synergistic or antagonistic effects, thus provide deeper insights into the environmental impact of chemical mixtures (Knapen et al., 2018; van der Oost et al., 2020).

To make AOPs practically useful for identifying immunotoxic substances, there needs to be a clear connection between the mechanistic insights provided by AOPs and the actual test methods and strategies used to detect and assess immunotoxicity in chemicals. However, the application of AOP frameworks to immunotoxicity is currently limited, which poses challenges to the development of effective assessment methods, particularly non-animal alternatives. New Approach Methodologies (NAMs), which includes non-animal testing methods, such as *in vitro* bioassays, computational models, and high-throughput screening, can play a critical role in bridging these gaps by providing the tools to assess key events within AOPs without the need for animal testing. NAMs can generate data on molecular and cellular responses which are critical for the construction or validation of AOPs, thereby strengthening their predictive capabilities. Although NAMs are not yet fully integrated into immunotoxicity assessments, they are increasingly being considered to fill these gaps. For example, the European Food Safety Authority (EFSA) recently conducted a study to investigate the immunotoxicity of PFAS using NAMs (EFSA, 2024). In addition, the EU's PARC[2] project is working to improve AOPs related to immunosuppression and other immune responses (Snapkov et al., 2024), while the OECD also contributes to AOP development for immunotoxicity (OECD, 2024) (see Table 1). Notably, the first AOP developed focused on contact allergy, for which several robust NAMs have been established. Furthermore, AOP has been proposed for inflammation, which is an important biological process involved in many target organ toxicities. Inflammation represented as a series of key events within AOPs, facilitates the integration of inflammation-related pathways and enhance the understanding of immune responses (Villeneuve et al., 2018).

In the context of drinking water, low exposures to contaminants for longer periods can lead to MIE, that may contribute to KEs leading ultimately to adverse outcomes. For example, the adverse effects of organohalogen disinfectant by-products (DBPs) (chloroform, trichloroacetic acid, and trichlorophenol) in drinking water have been linked to mitochondrial toxicity (McMinn et al., 2019). The MIE in this case is the excessive production of reactive oxygen species (ROS), triggered by DBPs, which activates two key pathways; (1) oxidative stress leading to mitochondrial DNA damage, and (2) glutathione depletion causing calcium dysregulation. Both lead to mitochondrial permeability transition pore (mPTP) opening, cytochrome c release, apoptosis, and mitochondrial disease, aiding in toxicological assessments and biomarker identification.

Although AOPs may seem too technical and unrelated to the daily operations of drinking water companies, having a basic knowledge of key concepts like MIEs and KIEs which trigger an adverse effects can be highly useful in guiding practical decisions for risk assessment. For instance, Effect-Based Monitoring (EBM) has gained recognition as a valuable approach for evaluating drinking water quality (Neale et al., 2023). EBM refers to a set of bioanalytical tools (bioassays) that assess water quality by capturing the mixture effects of many (known/unknown) chemicals present in water (Neale et al., 2022) (see Chapter 5). This approach is particularly important given the complex mixtures of chemical contaminants found in water bodies, which traditional targeted chemical analyses may not adequately capture. Knowledge of AOP processes can aid in identifying the most relevant effect-based method to detect immunotoxic effects of chemicals mixtures. In addition, it can support the establishment of effect-based trigger values (EBTs), which can be used as benchmarks to assess potential health risk, and can guide regulatory decisions to ensure safe drinking water. This, in turn, will allow water companies to implement more focused and efficient monitoring strategies. Even when time, budget, or resource constraints are present, prioritizing bioassays based on AOPs may ensure that testing targets the most critical indicators of immunotoxicity. An illustration of various events involved in immunotoxicity leading to immune-related AO is depicted in Figure 4 and a detailed explaination is presented in Appendix I.

[2] The Partnership for the Assessment of Risks from Chemicals (PARC) is dedicated to advancing next-generation chemical risk assessment to protect human health and the environment. PARC aims to provide innovative data, knowledge, methodologies, tools, expertise, and collaborative networks, to supports the European Union's "Chemicals Strategy for Sustainability" and contribute to the European Green Deal's "Zero Pollution" ambition Partnership for the Assessment of Risks from Chemicals | Parc.

Table 1. *Adverse outcome pathways (AOP) on immunotoxicity (source: of OECD (source: https://aopwiki.org/), MIE = Molecular initiating event; AO = Adverse outcome*

ID	Title of AOP	MIE	AO	OECD Status	References
14	Glucocorticoid Receptor Activation Leading to Increased Disease Susceptibility	Activation, Glucocorticoid Receptor	Increased, Disease susceptibility	Not defined	Lalone et al., https://aopwiki.org/aops/14
84	Suppression of immune system contributes to impaired development and leads to colony loss/failure	Not defined	Decrease, Number of worker bees, impaired, Hive thermoregulation Death/Failure, Colony	Not defined	Lalone et al., https://aopwiki.org/aops/84
85	Suppression of immune system contributes to abnormal foraging and leads to colony loss/failure	Not defined	Decreased number of worker bees; Death/Failure, Colony	Not defined	AOP-Wiki (aopwiki.org)
39	Covalent Binding, Protein, leading to Increase, Allergic Respiratory Hypersensitivity Response	Covalent Binding, Protein	Increase, Allergic Respiratory Hypersensitivity Response	Under Development	https://aopwiki.org/aops/39
40	Covalent Protein binding leading to Skin Sensitization	Covalent Binding, Protein	Sensitization, skin	Endorsed	https://aopwiki.org/aops/40
154	Inhibition of calcineurin activity leading to impaired T-cell dependent antibody response	Inhibition, Calcineurin Activity	Impairment, T-cell dependent antibody response	Endorsed	Komatsu, H., et al. (2021),
277	Impaired IL-1R1 signaling leading to Impaired T-Cell Dependent Antibody Response	Impaired IL-1R1 signaling in T cell	Impairment, T-cell dependent antibody response	Endorsed	Kimura, Y., et al. (2023)
314	Binding to estrogen receptor (ER)-α in immune cells leading to exacerbation of systemic lupus erythematosus (SLE)	Binding to estrogen receptor (ER)-α in immune cells	Exacerbation of systemic lupus erythematosus (SLE)	Under development	https://aopwiki.org/aops/314
315	Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response	Inhibition of JAK3	Impairment of T-cell dependent antibody response	Under Development	Yasuhiro et al. https://aopwiki.org/aops/315

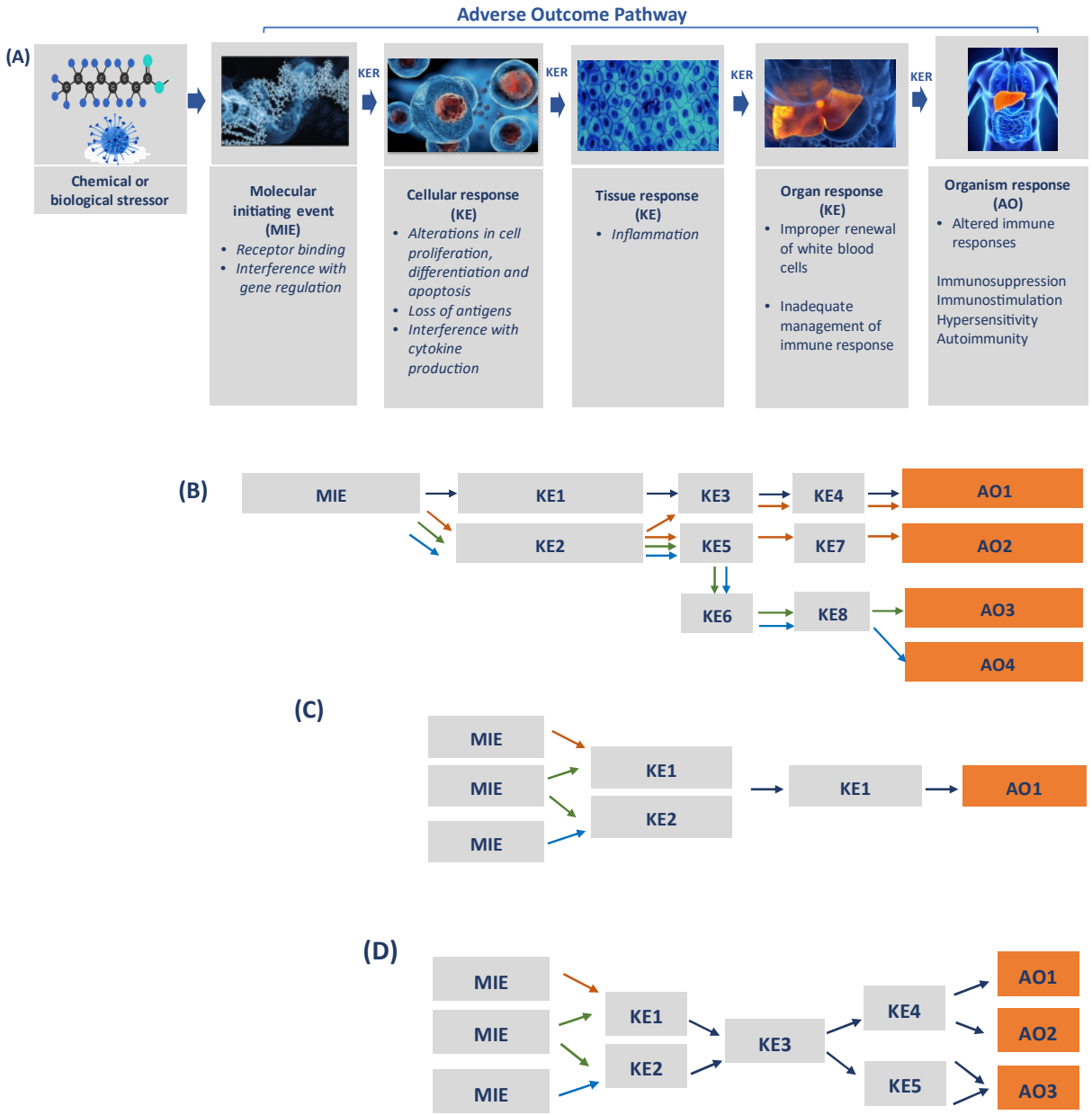

Figure 4: *Schematic representation of (A) an adverse outcome pathway (AOP). When multiple AOPs share common MIEs or pathways, they can be interconnected to form an AOP network; (B) a divergent AOP network in which a single MIE leads to multiple KEs, resulting in a variety of adverse outcomes; (C) convergent AOP network in which different MIEs lead to the same downstream event or adverse outcome; (D) A bow tie (complex) AOP network in which multiple upstream pathways converge at one or more KEs, and from there the network diverges into several downstream events or adverse outcomes. AOP starts with a Molecular Initiating Event (MIE) and progresses through a sequence of Key Events (KEs) occurring at different biological levels (cellular, tissue and organ). The pathway ends in an adverse outcome (AO) within the organism. The stressor represented by the chemical exposure, is not part of the AOP itself (Source: Knapen et al., 2018; Nymark et al., 2021; Finlayson et al., 2022).*

4 Overview of immunotoxicological testing options for hazard assessment of (drinking) water and food

4.1 Immunotoxicity testing

Immunotoxicity testing involves use of appropriate methods to assess immunological functions in order to understand the complex interactions between immune system and foreign substances, and any disruption in these interactions (Jamie et al., 2018). Extensive research in the field of immunotoxicology has resulted in the development of a range of *in vivo* and *in vitro* assays for the detection of immunotoxicants and identification of sensitive endpoints for assessing the effects on the immune responses (Hastings, 2018; Jamie et al., 2018; Naidenko et al., 2021). Immunotoxicity assessments are complicated due to its interactions with endocrine and neurological system which can complicate the interpretation of effects. While immune responses can be reliably detected in animal models, and their corresponding *in vitro* tests are well-established, replicating the adaptive specificity[3] of the immune system *in vitro* remains challenging. Nevertheless, recent advancements in NAMs provide a timely opportunity to introduce immunotoxicity assessments into broader testing frameworks. These advancements enable a more comprehensive evaluation of immune responses, overcoming previous limitations and enhancing the integration of immunotoxicity in toxicological assessments.

Several regulatory authorities have established specific guidelines for immunotoxicity testing to ensure the safety of chemicals and products across different sectors, including pharmaceutical, industrial and environmental chemicals (USEPA, 1998; ICH, 2005; OECD, 2023). In addition to the specific OECD Test Guidelines for skin sensitizing activity (*in vivo* OECD TG 406, *in vivo* TG 429, *in vivo* TG 442A-B, *in vitro* TG 442C-E, *in silico/in vitro* GL 497), other OECD guidelines for toxicity testing include assays for assessing immunotoxic potential in the context of more general toxicity testing, such as the 28-Day Repeated Dose Toxicity Study (OECD 407), the 90-Day Repeated Dose Toxicity Study (OECD 408), and the Extended One Generation Reproduction Toxicity Study (OECD 443). Key Targets in chemical-induced immunosuppression and *in vitro* test opportunities described by OECD, 2022 (OECD, 2022a) are presented in Table 2. The World Health Organisation/International Programme on Chemical Safety (WHO/IPCS) has published the Guidance for Immunotoxicity Risk Assessment for Chemicals (IPCS Harmonization project No. 10) oriented at immunosuppression, inadvertent immunostimulation, and autoimmunity caused by chemical exposure (see: pdf (oecd.org).

In addition to basic assays, computational methods including Quantitative Structure-Activity Relationship (QSAR) and read-across are valuable tools for predicting chemical toxicity and are increasingly used for predicting properties of chemicals for hazard identification and hazard characterization in the absence of experimental toxicity data (Benfenati et al., 2019; Cronin et al., 2022). QSAR is a computational modeling technique that establishes a mathematical relationship between the chemical structure of compounds and their biological activity or toxicity. The fundamental premise of QSAR is that similar chemical structures will exhibit similar biological properties. QSAR predictions are often employed in regulatory contexts to fill data gaps where experimental toxicity data may be lacking (Roy & Banerjee, 2024). While QSAR offer a valuable approach for predicting toxicity, and has been successfully applied to predict genotoxicity and endocrine disrupting effects, their robustness for predicting immunotoxicity is currently limited by challenges such as inadequate data, overprediction, false negatives, activity cliffs, and the complexity of incorporating factors like metabolism and individual susceptibility (Golden et al., 2023). However, this limitation does not apply to all areas of immunotoxicity. For example, QSAR models and datasets for contact allergens are well-established (Alves et al., 2015), and QSAR approaches are extensively used in the pharmaceutical industry for predicting toxic effects of drugs (Yang et al., 2023). To advance the field further, it is

[3] Ability of the adaptive immune system to recognize and respond to a virtually unlimited variety of specific antigens.

essential to incorporate QSAR models into immunotoxicity assessments, particularly for complex scenarios such as mixtures. Developing and refining customized QSAR models for these purposes will enable more robust screening and accurate predictions.

Read-across is a complementary approach that involves predicting the toxicity of a chemical (the target chemical) based on the known toxicological data of similar chemicals (source chemicals). This method relies on the assumption that structurally similar compounds will exhibit similar biological effects, thereby allowing for the extrapolation of toxicity data from one substance to another (Sikakana & Roberts, 2020). The read-across approach is often supported by expert judgment and can involve the use of various databases that compile toxicological information for numerous chemicals. The European Chemicals Agency (ECHA) has established guidelines for read-across methodologies, emphasizing the importance of demonstrating structural and functional similarity between the source and target chemicals (Arnesdotter et al., 2021). Other computational models, including mathematical models such as Universal Immune System Simulator (UISS) are available that enable advanced simulations of immune system and immunological processes (Pappalardo et al., 2022). The model which was initially developed for pharmaceutical domain can also simulate complex immune system interactions with environmental chemicals, providing a more holistic view of immunotoxicity than what QSAR models typically offer. For example, this tool successfully simulated the effects of PFAS exposure on the immune function (Pappalardo et al., 2022), hence providing a potentially useful tool for immunotoxicity assessment.

***Table 2**. Key Targets in chemical-induced immunosuppression and in vitro test opportunities (source: OECD, 2022).*

Key immunological targets (Tier)	*In vitro* opportunities	Cell model
Bone marrow (Tier 1)	Human lympho-hematopoietic colony-forming assay for myelotoxicity (e.g. CFU-GM)	Human bone marrow and umbilical cord blood; rodent bone marrow
Leukotoxicity (Tier 2)	Cell viability (e.g., MTT, LDH release assay, flow cytometry)	Rodent splenocytes; human peripheral blood mononuclear cells
Innate immunity (Tier 3)	NK cell activity	Rodent splenocytes; human peripheral blood mononuclear cells
	Monocytes/macrophages cytokines	Human peripheral blood mononuclear cells (e.g. whole blood assay); rodent splenocytes; cell lines (e.g. THP-1)
	Mast Cells/Basophils	Human basophils
Cell mediated immunity (Tier 3)	T cell proliferation	Rodent splenocytes; human peripheral blood mononuclear cells
	Mixed leukocyte response (MLR)	Rodent splenocytes; human peripheral blood mononuclear cells
	Cytotoxic T lymphocyte (CTL)	Rodent splenocytes; human peripheral blood mononuclear cells
	Cytokine production	Rodent splenocytes; human peripheral blood mononuclear cells (e.g. HWBCRA); human cell lines (e.g. Jurkat T cells)
	Transcriptomic profiles	Human peripheral blood mononuclear cells; human cell lines (e.g. Jurkat T cells)
	In vitro antigen presentation to T cells	Mouse cell lines (e.g. 3A9; Ch27B)
Humoral immunity (Tier 3)	B cell proliferation	Rodent splenocytes; human peripheral blood mononuclear cells
	In vitro antibody production	Rodent splenocytes; human peripheral blood mononuclear cells

4.2 Prospective approach for immunotoxicity testing for drinking water quality

Drinking water contaminants are generally present in low concentrations, yet chronic exposure to even small amounts of immunotoxic substances over time could lead to subtle, long-term impacts on the immune system. While such effects may not manifest acutely, they can gradually impair immune function. For example, impaired T-cell activation and functional responses have been observed in individuals as a result of chronic exposure to arsenic (As) in drinking water (Gonsebatt et al., 1994; Dangleben et al., 2013). Certain groups, such as children, the elderly, or individuals with compromised immune systems, may be more sensitive to lower levels of immunotoxic substances (Holsapple & O'Lone, 2012; Mlelwa et al., 2024). In these cases, even minimal exposure could have more pronounced effects. Moreover, the water sector is constantly dealing with new chemicals many of which remain insufficiently tested for immunotoxicity. Some of these substances may exhibit immunosuppressive or immune-activating properties at lower, environmentally relevant concentrations. Therefore, incorporating testing for such endpoints would provide early warnings about potential risks. Furthermore, given the complexity and variability of contaminants in drinking water sources, interactions among multiple substances may lead to immunotoxic effects, even if the individual components are not highly toxic.

As the science of immunotoxicity advances, regulatory agencies are likely to place greater emphasis on immunotoxic endpoints. Integrating immunotoxicity assays into water quality monitoring could therefore play a critical role in future-proofing water safety assessments. However, despite the growing recognition of immunotoxicity as an important endpoint, the absence of standardized protocols for immunotoxicity testing in environmental matrices, including drinking water, remains a significant challenge. Currently, in the testing of drinking water or surface water quality in the Netherlands, no assays for immunotoxicity are included (KWR 2019.040; Pronk et al., 2021). Moreover, there is no clear evidence that immunotoxic effects in drinking water or sources are up to a level where health could be at risk. In the current project, we did not focus on fully developing an approach for immunotoxicity testing for routine water quality monitoring, as it requires further refinement and validation of bioassays tailored to drinking water contexts. Addressing these challenges will necessitate future research and collaborative efforts. To support understanding, we provide a table summarizing the most commonly used immunotoxicity bioassays (see Table 4, Appendix II). However, the direct applicability of these assays for drinking water testing remains uncertain, as many require extensive validation. Such validation, though outside the scope of this project, is critical for establishing reliable methods for water quality monitoring.

As a pathway forward, we propose the development of a tiered risk assessment framework adapted from the existing OECD framework for immunotoxicity. This framework would be tailored to the unique requirements of drinking water testing. A tiered approach typically involves multiple levels of testing, beginning with initial screening assays to identify potential hazards and advancing to more specific and mechanistic studies to uncover the underlying immunotoxic mechanisms (Phelps et al., 2020).Several studies have highlighted the importance of implementing tiered approaches in assessing immunotoxicity to enhance the reliability and relevance of toxicity testing (Vos & Kuper, 2004; Phelps et al., 2020). While traditional immunotoxicity testing approaches often rely on animal models, such as, the T-cell-dependent antibody response (TDAR) and local lymph node assays (LLNA) (Anderson et al., 2011; Lebrec et al., 2014), these are not directly applicable for water testing. For practical purposes, the focus should shift toward New Approach Methodologies (NAMs), including i*n vitro* systems and alternative models like *C. elegans* (Yu et al., 2015; Li et al., 2021; Ramhøj et al., 2024). The advantage of using *C. elegans* is its potential to be developed into a rapid whole-organism assay, effectively capturing innate immune responses as a starting tier in the assessment. These alternative approaches provide promising tools for enhancing the relevance, speed, and applicability of immunotoxicity testing in the context of drinking water.

The current OECD framework focuses on immunotoxicity, particularly immunosuppression, and provides valuable guidance for evaluating chemicals (see Table 2). However, the adequacy of this framework in addressing the specific challenges of drinking water was not investigated in the current project. Since the scope of the current study does

not allow for a comprehensive evaluation of the existing OECD framework's applicability to the drinking water scenario, we propose a tailored framework as a follow-up research. This framework would retain the core principles of the OECD approach but incorporate modifications relevant to the characteristics and testing procedure of drinking water contaminants. Importantly, this tailored framework is recommended to prioritize the use of NAMs or, at a minimum, non-rodent models to align with ethical considerations and emerging regulatory trends. The aim is to ensure that the tiered approach remains scientifically sound while being better suited to the specific nature of water as an exposure route. This approach also positions the water sector to better protect vulnerable populations and respond to future regulatory shifts.

Key modifications to the OECD framework may include:

1. Addressing complex mixtures of chemicals and emerging contaminants, which are often present in drinking water and may require special consideration in terms of immunotoxicity risk assessment.
2. Integrating Quantitative Structure-activity relationship (QSAR) models, although current data on their performance for immunotoxicity endpoints are limited, and further investigation into their reliability is needed.
3. Assessing the sensitivity and compatibility of *in vitro* testing with water extracts to ensure practicality and accuracy in real-world scenarios.
4. Exploring the utility of alternative animal models, such as *C. elegans* and planarians, to determine their compatibility with water samples and their suitability for screening contaminants in drinking water sources. These organisms can serve as intermediates between *in vitro* and mammalian testing, helping bridge the gap between early-stage screening and more complex *in vivo* studies.

5 Immunotoxicological potential of contaminants in drinking water sources and its implications for drinking water companies

Monitoring water contaminants is essential to ensure the safety of water intended for human consumption. Although many contaminants are regulated and anticipated by drinking water companies, others remain undetected, unquantified, and toxicologically uncharacterized. This is particularly concerning for (potentially) immunotoxic substances, which can impair the immune system and compromise overall health. Assessing the risks of these contaminants is challenging, as immune system effects can be subtle, may not appear until after prolonged exposure, or may only impact vulnerable populations, making them difficult to detect and evaluate accurately. Thus, while all the classes of contaminants warrant attention, the unique and critical role of the immune system in health makes the monitoring of immunotoxic substances particularly important. The RIVM in the Netherlands has used the EFSA's health-based guideline value, TWI as the basis for their health assessments of PFAS and has proposed a drinking water guideline value in line with the EFSA's recommendations. Drinking water quality limits specifically based on immunotoxic potential are not common, as most existing regulations primarily focus on general toxicological endpoints such as carcinogenicity, reproductive toxicity, and organ-specific toxicity. Consequently, there are currently no established water quality limits that are exclusively based on immunotoxic effects. However, certain contaminants relevant to water quality, such as PFAS, are recognized for their immunotoxic effects, and have been considered in the development of drinking water limits. EFSA has established a Tolerable Weekly Intake (TWI) of 4.4 nanograms per kilogram of body weight per week (ng/kg bw/week) for PFAS, particularly focusing on four of the most prevalent and well-studied compounds: PFOS, PFOA, PFNA, and PFHxS (EFSA, 2020). This decision was informed by a

thorough review of the health risks associated with PFAS exposure, particularly concerning the immune system. Following the EFSA TWI, the National Institute for Public Health and the Environment (RIVM) in the Netherlands derived an indicative drinking water standard of 4.4 ng/L expressed as PFOA equivalent (see: perfluoroctaanzuur | Risico's van stoffen (rivm.nl) , ensuring that exposure through drinking water remains within safe limits while accounting for contributions from other PFAS sources. The last word has certainly not been said about the PFAS guideline values; recently a report challenges the clinical relevance of the immunosuppression effect found by PFAS (Reinikainen et al., 2024). Investigated PFAS (especially short-chain) did not show a strong response in ToxCast assays related to immunotoxicity (Naidenko et al., 2021) although this may also be allotted to the capacity of the assays to capture this effect.

For drinking water companies, understanding and managing the (immuno)toxicological potential of contaminants is critical to ensuring the safety and health of their consumers. Given the complexity of the immune system and the various mechanisms by which chemical contaminants may exert immunotoxic effects, there is a pressing need to identify, compile, and continuously update a list of immunotoxic substances in a drinking water context. This will help to prioritize monitoring efforts toward immunotoxic agents of emerging concern and enhance immunotoxic risk assessment and management actions. It may be perceived that immunotoxic effects typically occur at levels which are unlikely to be found in drinking water. As a result, the need for bioassays specifically targeting immunotoxicity in this context could be thought irrelevant. However, this viewpoint could overlook several important considerations, particularly when assessing the risk of low-level, long-term (mixture) exposure, the vulnerability of certain populations, and the increasing presence of emerging contaminants in drinking water sources and potential by-products from treatment process. While drinking water is usually safe, source water, which supplies drinking water is often more contaminated, and some disinfection by-products or other chemicals formed during treatment could be immunotoxic (Gao et al., 2015).

5.1 Water relevant compounds with their immunotoxic potential, and if relevant, associated other toxic endpoints

Many water contaminants are suspected or have been identified to possess immunotoxicological potential, particularly heavy metals (e.g., arsenic, lead), pesticides (e.g., atrazine, glyphosate), pharmaceuticals (e.g., antibiotics), and hormones (e.g., estrogen). Despite these known hazards, numerous water contaminants remain either poorly evaluated or completely unevaluated for their immunotoxic potential. This gap in knowledge is particularly concerning for the national and European Commitments in ensuring the sustained provision of safe drinking water. As the body of scientific evidence grows, it is increasingly clear that initial steps can be taken using existing data to monitor and signal immunotoxicity potential of (drinking) water micropollutants. This approach is crucial for safeguarding water quality and maintaining regulatory compliance.

An initial step in the present study involved collecting data from databases, such as IRIS (US EPA Chemical Search | IRIS | US EPA) and the ECOTOX Knowledgebase (US EPA ECOTOX | Home (epa.gov), Last update 14 March 2024). However, given the vast number of chemicals listed in these databases, it was impractical to evaluate the immunotoxic potential of each chemical individually within the scope of the current project. Alternatively, we focused on identifying the 500 chemicals that overlapped among the IRIS, ECOTOX, and the Dutch REWAB database (REWAB - KWR (kwrwater.nl) to ensure that these compounds were present in drinking water sources., The method involved filtering data from the IRIS and ECOTOX databases based on the specific toxicological endpoint of immunotoxicity, yielding 29 hits from IRIS and 484 hits from ECOTOX, resulting in a combined total of 513 immunotoxic substances.

With this list of identified immunotoxicants from the toxicological databases, the next step was to pinpoint those most relevant to water quality. To achieve this, we cross-referenced the 513 substances with the REWAB database, which contains information on 1,869 substances related to water quality. Through this comparative analysis, we identified 247 substances in REWAB that are classified as immunotoxic, highlighting their relevance to water

monitoring and risk assessment. (Table 5, see Appendix III). These chemicals primarily include plant protection products (PPP), industrial chemicals and pharmaceuticals. With the predominant presence of pesticides within the compiled list of potentially immunotoxic substances, prioritizing the elucidation of immunotoxicity risks of PPPs inherent in water samples seems warranted. This is also emphasized by the significant contribution of pesticides to anthropogenic contaminants detected in Dutch groundwater, the main source (60%) of drinking water (van Loon et al., 2020).

In an extra analyses, it was determined which chemicals, tested in at least 5 immunotoxicity related ToxCast assays (Appendix IV) were relatively potent (in the 50% most potent chemicals tested per assay) AND were present in the REWAB database. This yielded another 24 substances. As the assessment only includes currently monitored substances, there may be immunotoxic chemicals that are not yet monitored or recognized as threats. These "unknowns" remain a critical concern and represent a gap in our understanding and regulation of water contaminants. To address this, incorporating data from broader scientific literature or databases could help identify additional substances with immunotoxic potential that are not yet captured in current surveillance frameworks. These sources could provide insights into emerging contaminants, including pharmaceuticals, microplastics, and novel industrial compounds that may have immunotoxic effects but are not part of routine monitoring.

The immunotoxicological classification of these substances is generally established by the authors of original studies. To ensure accuracy, we undertook a comprehensive review of the list of substances. However, given the impracticality of thoroughly evaluating all 247 substances within the current project, we implemented a prioritization strategy by focusing on substances that exceeded the reporting limit, i.e. the lowest detectable concentration (representing a worst case scenario out of all locations where they were detected). This approach allowed us to narrow down the list to 49 substances for in-depth review. From this refined list, we examined both experimental and epidemiological data to assess their immunotoxic potential. Our findings show that about 35 of these substances belonging to different chemical categories exhibited immunotoxic properties. Additionally, some of these substances were found to have potential adverse effects on several other endpoints. The detailed results of our findings are presented in Table 5 (see Appendix III).

It is worth noting that while this study highlighted 247 chemicals, other contaminants listed in REWAB database could possess similar properties. Furthermore, there may be new or unknown substances (CEC's) in the water system that have yet to be identified which may also have immunotoxic potential. Therefore, further research is necessary to explore the immunotoxic potential of these additional or emerging substances, which was beyond the scope of the current project This knowledge is crucial to enhance our understanding of the full spectrum of immunotoxic risks posed by drinking water contaminants and to ensure protection of public health. Although the main toxicity endpoint of the current study was immune system as it is a known sensitive target for pollutants (Wang et al., 2022), other toxic endpoints may also be associated with immune system damage via (dis)similar mechanisms (Vidal et al., 2022). For example, liver toxicity caused by PFOS may be associated with inflammatory cell infiltration, a process by which immune cells infiltrate neighboring tissues and cause inflammation (Lin et al., 2022; Wang et al., 2023). We have included such associations in our analysis table, indicating if any other toxic endpoint is linked to immune damage or is caused as a consequence of exposure to the specific chemical (Table 5. see Appendix). This comprehensive approach allows for a more thorough understanding of multiple impacts of these contaminants.

Potential toxic activity of other existing and new chemicals relevant to water quality can also be assessed through exploration of shared chemical structures and properties among these pollutants. Approaches such as QSAR and read-across methodologies could facilitate this task. While the application of (Q)SARs in evaluating physicochemical attributes and some environmental toxicities has gained regulatory traction, their utility in assessing complex toxicological traits remains limited. As noted by the European Chemicals Agency (ECHA, 2016), (Q)SARs cannot replace experimental toxicological studies for classification, labelling, or comprehensive risk assessments.

Nonetheless, they can be integrated with complementary datasets within a weight of evidence framework to enhance decision-making processes.

5.2 Overview of options and gaps in effect-based testing for immunotoxicity including availability of trigger values.

Effect-based methods (EBMs) also known as effect-based monitoring are bioanalytical techniques that utilize the responses of whole organisms (*in vivo*) or cellular bioassays (*in vitro*) to detect and quantify the effects the combined effects of known and unknown chemicals in a range of water samples including drinking water, surface water, wastewater, recycled water, and stormwater (Brack et al., 2019; Neale et al., 2023). While targeted analysis can identify specific chemicals, it often fails to account for the biological effects of complex mixtures, especially when individual chemicals are present at low concentrations (Neale et al., 2023). The implementation of effect-based methods is supported by various regulatory frameworks, such as the European Water Framework Directive, which encourages the use of bioassays as part of comprehensive water quality monitoring strategies (Brack et al., 2019).
In general, when selecting a bioassay either for testing a chemical or an environmental sample, a number of factors need to be considered to ensure its suitability and effectiveness. The factors include, its sensitivity and specificity, throughput, validation, and regulatory acceptance (Basketter et al., 2012). Sensitive and specific bioassays are essential for detecting low concentrations of toxicants and distinguishing between different types of toxicants. High-throughput assays are desirable for screening large numbers of samples (Schilter et al., 2019). Practical considerations such as costs, ease of use, and availability of equipment and expertise need to be considered. In addition, using validated bioassays ensures that the results are reliable and can be compared across studies (Schilter et al., 2019). Furthermore, regulatory acceptance ensures that the assay meets the required standards set by regulatory bodies (Basketter et al., 2012).

A bioassay response does not inherently indicate poor water quality, it must be evaluated against an Effect-Based Trigger value (EBT) to determine its significance (Neale et al., 2023). EBTs serve as thresholds to distinguish acceptable from unacceptable biological responses in water samples based on observed effects. These thresholds are often expressed as Bioanalytical Equivalent Concentrations (BEQ) reference compound that elicits a similar biological response in a bioassay, enabling direct comparisons between the measured bioassay effects and the EBT (Escher et al., 2015; Been et al., 2020). The concept of BEQ is based on the idea that if chemicals in a mixture share the same mode of action, their combined effects can be described by the concentration addition concept (Been et al., 2020). When the BEQ is lower than the corresponding EBT, the water quality is considered acceptable for the endpoint being assessed. Whereas exceedance of BEQ to the corresponding EBT, warrants further investigation. However, it is important to note that an exceedance does not necessarily imply an immediate risk; it merely indicates a hazard that requires additional assessment. Such actions may include retesting the sample, conducting targeted chemical analyses, or optimizing water treatment processes to mitigate the identified risks.

When selecting a bioassay to assess toxicity, several factors should be considered. One key aspect is whether the assay is sensitive enough to detect the most relevant immunotoxicants in the water system. In addition, the availability EBT value is important. With a trigger value assay responses can be interpreted in two categories: 'safe to use' or 'risk not excluded'. Another important factor that is relevant for choosing a bioassay for testing water samples is sample enrichment. Adequate sample pretreatment to enrich the target analytes is necessary for detecting trace levels of pollutants. Enrichment processes significantly impact the sensitivity and reliability of bioassays by concentrating target analytes or effects present in samples, thereby enhancing the detection capabilities of the assay (Escher et al., 2012). This is relevant for any type of environmental sample where analytes may be present at low concentrations. However, if contaminants are present in high concentrations, or if highly sensitive testing methods are used, enrichment may not be necessary, as the substances will already be detectable at their natural levels. In the context of water quality monitoring through bioassays, sample enrichment plays a critical role in enhancing the sensitivity of toxicity testing (Muller et al., 2007). Studies that combined passive sampling with toxicity testing

observed distinct improvements in sample quality following sample enrichment processes, leading to more accurate assessments of genotoxicity and other biological responses (Muller et al., 2007).

Although EBTs have been established for various bioassays, including estrogenic activity, glucocorticoid activity and oxidative stress for different water types (Escher et al., 2015; van der Oost et al., 2017; Been et al., 2021), the lack of a standardized approach to deriving these thresholds introduces variability in their application. This inconsistency can cause confusion among regulators and water quality managers regarding the appropriate EBT to use in specific contexts. Furthermore, EBTs are typically bioassay-specific, meaning that each endpoint requires its own tailored EBT rather than a generic threshold. This specificity can complicate result interpretation, particularly when multiple bioassays are employed for comprehensive water quality assessments. Additionally, while EBTs aim to protect human and ecological health, their effective application relies on careful contextualization. There is a growing need for comprehensive guidelines to ensure EBTs are appropriately developed and used for specific endpoints, such as immunotoxicity, across diverse water types. The evolving knowledge surrounding EBTs and their integration into regulatory frameworks poses challenges to their broader acceptance and consistent implementation. These challenges are particularly pronounced for endpoints like immunotoxicity, where further research and standardization are required to enhance their role in water quality monitoring programs.

To derive such a trigger value for immunotoxicity, health based guideline values (pGLV) and bioactivity data on individually tested immunotoxic substances in the assay should be available (Been et al, 2021). Bioactivity data for assays in Europe are not centrally collected, although a database for bioactivity data is currently under construction in the European NORMAN network (https://www.norman-network.com/nds/badb/). Finding bioactivity data from, for instance, scientific literature is a potential bottleneck for deriving trigger values. In a second report (KWR 2025.004) we describe how trigger values for any data-poor assay of choice can be derived by borrowing information of other, similar assays.

ToxCast database contains bioactivity data for many assays with immunotoxic relevance (Naidenko et al., 2021). Although the specific assays are generally not in use in Europe, it is a relevant source to see if any of the tested endpoints in the assays would in theory react sensitively to the immunotoxicants identified in paragraph. This would help direct the choice of a relevant assay for immunotoxicity for Dutch drinking water. For this purpose, it was investigated if there was any immunotoxic relevant assay in ToxCast (see Appendix IV) which would be able to signal all substances in Table 6 (see Appendix IV). For this analysis we assumed that the substances would need to have an active response ('hit') in ToxCast. Results showed that only six substances were tested, over all assays (Bisphenol A, Carbendazim, Silica, Simazine, Imidacloprid, (E)-1,2-Dichloroethylene). This expectedly is a result of the focus of ToxCast on industrial or food relevant substances; it was not intended for water quality monitoring. There was no assay in which all substances (with a maximum of 6) either were tested or showed a response in the assay. This means for now a potential candidate to accurately signal the Dutch water relevant substances cannot be pointed out. It is possible that the mode of action of the six chemicals is different and different assays are required. Further research can point this out. Two assays did contain data and a response for more than one of the six substances, one related cytokines modifying macrophage function BSK_LPS_MCSF_down (Bisphenol A and Carbendazim) and one targeting the NFKB1 receptor, LTEA_HepaRG_NFKB1_dn (Carbendazim and Imidacloprid).

6 General Discussion and Future Recommendation

6.1 General Discussion

The focus on identifying, monitoring, and assessing immunotoxic water contaminants is crucial for protecting public health. While regulatory frameworks and testing guidelines provide a foundation, ongoing research and updated risk assessments are necessary to address emerging concerns and ensure the safety of drinking water. Scientific and regulatory authorities are increasingly recognizing the critical importance of assessing the impact of chemical

contaminants on the immune system. Research indicates that even minimal levels of contaminants like pesticides, endocrine disruptors, and disinfection by-products can disrupt immune function, posing significant health risks (As et al., 1999; Bansal et al., 2017; Christin et al., 2003; Milla, 2011). This is particularly concerning in the context of drinking water, where trace amounts of pollutants may persist despite treatment efforts (Arp et al., 2017; Eke et al., 2020), raising concerns about the potential long-term health effects of continuous exposure to low levels of contaminants, especially for vulnerable populations (children, the elderly, and individuals with pre-existing health conditions). Impaired immune function increases the risk of infectious diseases and certain cancers in humans in particular in sensitive groups as well as animals, necessitating inclusion of immunotoxicity endpoints in risk assessments for a more thorough evaluation of the health risks, which may also be relevant to drinking water contaminants (Frawley et al., 2016; Quinete & Hauser-Davis, 2020; Sweet & Zelikoff, 2001). Evaluation of this endpoint, can help to determine the health effects that traditional regulatory toxicity testing methods may overlook, thereby improving the overall health risk assessment process (Sweet & Zelikoff, 2001; Frawley et al., 2016).

The European Union's Drinking Water Directive does not specifically address immunotoxicity, although it incorporates health-based guidance values from the World Health Organization for certain substances partially based on immunotoxic effects. Recent scientific evidence has synthesized key characteristics of agents influencing immune processes, providing a useful framework for risk assessment of immunotoxicity. This framework is particularly relevant for chemicals of emerging concern with immunotoxic effects, such as PFAS, due to their widespread and persistent presence in the environment and water sources (Bline et al., 2024). In September 2023, the European Parliament's Committee on the Environment, Public Health and Food Safety adopted a report requiring the European Commission to consider introducing hazard criteria for immunotoxicity and neurotoxicity by December 2025. This report also calls for the adoption of delegated acts where appropriate and the promotion of these hazard classes at the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UNGHS). As of now, the delegated acts related to immunotoxicity are currently under consideration. The Commission is expected to address this matter as part of its ongoing revision of the CLP Regulation, with the timeline set for 2025.

In this report, existing databases were examined to identify substances routinely monitored in drinking water in the Netherlands that have been classified as immunotoxicants, mainly pesticides and industrial chemicals. This provides important insight into the potential immunotoxic profile of national drinking water samples. The prevalence of these chemicals in the compiled list of immunotoxins suggests that drinking water companies may focus on immunotoxicity assays that target key immune effects. These assays should be incorporated into EBM strategies, which allow water utilities to assess the biological effects of chemical mixtures in real-time (Busch et al., 2016; Neale et al., 2022). Since EBM does not rely solely on identifying specific compounds but instead evaluates the overall biological impact, which helps detect the effects of unmonitored chemicals that may be contributing to immunotoxicity. This is particularly relevant for emerging contaminants or newly identified immunotoxicants, allowing water utilities to stay ahead of potential public health risks. We propose adopting a mixture toxicity approach using bioassays, such as those based on nematode *C. elegans* or planarians (*Schmidtea mediterranea*). These organisms offer valuable models for high-throughput screening of complex mixtures, as they are sensitive to a variety of toxicological pathways, including immunotoxicity.

Advanced approaches, such as AOPs, offer a structured framework that connects MIEs to adverse outcomes, and can facilitate evaluation of immunotoxicity (Bal-Price et al., 2015). Assessing MIEs often requires the use of advanced methods, such as *in vitro* methods to measure the key biological events that occur along the AOP. By selecting tests that target specific key events along the AOP relevant to immunotoxicity can help to better evaluate the short-term and long-term consequences of chemical exposure on the immune system (McMinn et al., 2019; Mazein et al., 2023). In the context of drinking water, AOPs can play a crucial role in identifying key biomarkers and pathways to understand how specific substances in drinking water can disrupt the immune system and may contribute to chronic health effects. Furthermore, the knowledge obtained from AOPs can help in selection of appropriate endpoints and bioassays for effect-based monitoring. Effect-based monitoring of water quality is highly useful to inform hazard

assessment (Dingemans et al., 2019). Applying these methods enables the interpretation of responses induced by water samples containing (low-level) chemical mixtures of known and unknown compositions, which cannot be addressed by chemical analysis alone (Dingemans et al., 2019; Neale et al., 2023). While effect-based monitoring methods are valuable, it is essential to acknowledge the uncertainties they entail related to risk interpretation. Therefore, a diverse array of *in vitro* bioassays (primarily mammalian cell models), and well plate-based *in vivo* assays (small organisms) in combination with analytical chemistry are recommended for water quality assessments and to improve the efficacy of effect-based monitoring methods and interpretation using effect-based trigger values (Dingemans et al., 2019; Escher et al., 2023; Neale et al., 2023).

Drinking water companies may also consider new high-throughput detection systems applying multi-biomarker approaches to evaluate the immunotoxicity potential of contaminants at water abstraction points. For example, it has been demonstrated that surface water extracts from a riverine site near an industrial area, showed immunosuppression and immunomodulation effects (Li et al., 2022). Drinking water companies take care that their treatment methods prevent contaminants of emerging concern from ending up in drinking water in e.g. in instances of peak water source pollution. Furthermore, focus and concern is continuously growing beyond the exposure levels to the hazardousness of CEC (e.g., endocrine disrupting chemicals, immunotoxic agents) at low levels, in mixtures, and for extended periods of time, and if they exert or exacerbate adverse effects in the immune system (Quinete & Hauser-Davis, 2021). Parallel measures of immunotoxicity in the finished water product by incorporating EBM and/or targeted immunotoxicity assays at key could provide valuable insights into the effectiveness of drinking water production methods in reducing immunotoxicological potential. Large national initiatives aimed at developing new approach methodologies, such as the Virtual Human Platform for Safety Assessment project (VHP4Safety) (https://www.nwo.nl/en/projects/nwa129219272), where KWR plays an active role, have significant potential to accelerate the evaluation of complex acting molecules, including immunotoxicants. By leveraging *in vitro* and *in silico* models, the consortium integrates essential and complementary expertise to design, test, and refine the VHP4Safety platform. This collaborative effort aims to provide a robust, future-oriented framework for safer drinking water practices.

A detailed evaluation of the immunotoxicity endpoints affected by many chemicals identified in present study remains incomplete. Establishing whether alterations in immune markers equate to actual adversity in immune system function is challenging, partly due to significant variations with age, species, sex, and assessment methods across studies (EFSA, 2015). Furthermore, the immune system's redundancy mechanisms, which compensate for compromised parts, complicate the establishment of standardized criteria for immunotoxic classification and interpretation of experimental adverse effects. Although for most groups of the general population, the intake of drinking water contaminants may not represent a health concern, it must be considered that other people may be more vulnerable (e.g., immunocompromised) or have a weakened immune system (e.g., pregnant women, infants, elderly). The EU's Chemicals Strategy for Sustainability places a strong emphasis on protecting vulnerable populations, including pregnant, nursing, children, infants, unborn and the elderly, as well as workers and residents subject to high and/or long term chemical exposure by reducing their exposure to the most hazardous chemicals to safeguard their health and well-being (EU Commission, 2020).

In conclusion, consideration of immunotoxicity as a key aspect of drinking water quality assessment is critical for preserving drinking water quality, especially given the potential long-term effects of chemical contaminants on the immune system. The integration of advanced approaches, such as, AOPs-based testing for immunotoxicity assessment and effects-based monitoring will enrich our understanding of potential hazards that contaminants in drinking water sources may possess. Furthermore, this will allow drinking water companies to better manage risks and ensure the safety of drinking water for all.

6.2 Recommendation and follow-up research

To address the limitations of conventional monitoring techniques in detecting specific immunotoxic effects, particularly those induced by emerging contaminants and complex mixtures in drinking water sources, there is a pressing need to incorporate immunotoxicological information on individual substances and integrate immunotoxicity testing methods into the routine evaluation of drinking water quality. In this context, some of the important recommendations for follow-up research on immunotoxicity are outlined below.

1. Future studies should integrate chemical and microbial risk assessments, especially with the growing focus on water reuse, to ensure timely identification of immunotoxic effects and safeguard public health. Due to limited resources, our study focused on assessing the immunotoxic potential of select drinking water chemicals. Given the vast array of monitored and emerging substances, further research is needed to evaluate their immunotoxicity, including chemical mixtures and interactions with microbial contaminants.

2. It is recommended to explore the relationships between immunotoxicity, neurotoxicity, and endocrine toxicity, using simple *in vivo* models like nematodes (*C. elegans*) and/or planarians (*S. mediterranea*). It is also recommended to assess the potential of these simple models for routine immunotoxicity testing by examining their response to contaminants found in drinking water and and their suitability for high-throughput screening, standardized protocols, and effect-based water quality monitoring.

3. Implementation of a tiered approach to testing, starting with broad screening bioassays and moving to more detailed studies on high-risk contaminants will help prioritize which chemicals to focus on, based on their potential to affect immune health.

4. Establish EBTs for chemical mixtures with immune effects. EBTs are the thresholds that indicate whether a chemical concentration requires further action. This will enable quicker decision-making when assessing water safety using effect-based methods.

5. Prioritize substances not routinely tested for immunotoxicity, from sources, such as chemical industries, pharmaceuticals, and microplastics, based on factors like environmental persistence, potential for human exposure, and possible health risks.

6. Develop consistent, scientifically validated testing protocols for immunotoxicity aligned with both Next Generation Risk Assessment (NGRA) and water quality monitoring, to ensure that practices reflect the latest advancements in immunotoxicological science.

7. Given the complexity of immunotoxicology, collaboration with laboratories and research institutions can aid in developing testing protocols, improving bioassay interpretation, and risk assessments. Simultaneously, investing in training and capacity building at KWR will strengthen our capabilities and support assessments of immunotoxic substances in water quality monitoring.

We propose a follow-up project to develop a testing framework for immunotoxicity, combining traditional and advanced approaches to ensure a more accurate and relevant evaluation of water-relevant contaminants, with a focus on developing standardized immunotoxicity bioassays for drinking water.

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I Appendix: Adverse Outcome Pathways (AOPs)

Definition AOP: Refer to chapter 3 of this report.
A description of AOPs focusing on immunotoxicity is provided below.

Molecular initiating event (MIE)
MIE represent the initial interactions of a stressor (such as a chemical or a pathogen) with a molecular target through receptor binding, protein binding, enzyme inactivation, or DNA damage (USEPA, 2023). These initial interactions trigger a sequence of events, leading to adverse outcomes at the organism level (Perkins et al., 2019). MIEs can be classified either as specific or non-specific. Specific interactions are characterised by targeted interaction of a stressor with a particular receptor, whereas non-specific interactions involve interactions with various cellular proteins (Tirumala et al., 2021). Several examples of MIEs in AOPs associated with immunotoxicity can be found in literature. For example, in the context of skin sensitization, covalent binding to proteins has been identified as a high confidence MIE that ultimately leads to skin sensitization (Perkins et al. 2015). Aryl hydrocarbon receptors (AhR) have been suggested to serve as a potential MIE for the immunotoxicity of dioxin-like chemicals (Segner et al., 2021). Studies have demonstrated that the interplay between AhR and NF-κB (a major transcriptional factor regulating genes of innate and adaptive immune response) exert a substantial influence on various pro-inflammatory signals (Kreitinger et al., 2016). Furthermore, the formation of protein corona (a layer of biomolecules, usually proteins) has been identified as the most suitable MIE that is relevant for nanomaterials (Hofer et al, 2022).

Cellular responses (Key Events: KE)
Cellular responses associated with immunotoxicity include a wide array of changes at the cellular level induced by chemical perturbation leading to adverse outcomes at the organismal level. These responses include changes in cell functionality which potentially manifest as inhibition (immunosuppression) or stimulation (immunostimulation) of cytokine production (Côté-Maurais et al., 2014). Cytokines, which are the messenger molecules of immune system, play a crucial role in mediating cellular responses of innate and adaptive immunity (Abbas et al., 2012) (see Table 3). Pro-inflammatory cytokines, such as Tumour necrosis factor (TNF), Interleukin -1 (IL-1) and Interleukin-6 (IL-6) are important for innate immune system and act on different target cells. They are primarily released from tissue macrophages and mast cells, with other sources including endothelial and epithelial cells (Abbas et al., 2012; Cavaillon and Adrie, 2009). These cytokines can directly affect tissues or activate secondary mediators to initiate the coagulation cascade, potentially leading to organ dysfunctions through specific and nonspecific damage to the tissues (Sitia et al., 2010; Flammer et al., 2012; Šibíková et al., 2018). Environmental pollutants, such as nanoparticles (NPs) like Zinc oxide (ZnO) NPs have been reported to induce inflammation through cytokines and chemokines, (Kim et al., 2014; Bi et al., 2023). Furthermore, exposure to metals like lead (Pb), cadmium (Cd), and mercury (Hg) has been shown to induce immunomodulatory effects. For instance, lead has been demonstrated to selectively induce T-helper 2 (Th2) and M2 macrophages, altering both humoral and cellular immune responses (Kasten-Jolly & Lawrence, 2014; Bou Zerdan et al., 2021). Similarly, cadmium and mercury exhibit immunomodulatory effects, with $CdCl_2$ and $HgCl_2$ inhibiting RNA, DNA, and antibody synthesis, exerting early inhibitory effects on B cell activation (Daum et al., 1993; Bou Zerdan et al., 2021). Furthermore, exposure to endocrine disrupting chemicals, such as tributyltin, has been shown to impact immune response, antioxidant defense, and immune gene expression in various species toxicants (Da Silva et al., 2023).

Tissue and organ responses (Key Events: KE)
Exposure to immunotoxic substances can cause various forms of tissue damage and dysregulation, including inflammation (Villeneuve et al., 2018). Inflammation, clinically characterised at the tissue level by redness, swelling, heat, pain, and loss of function involves a complex cascade of interactions among pro- and anti-inflammatory mediators (Megha et al., 2021). While inflammation normally serves as an adaptive response to various stressors, xenobiotics can induce prolonged or severe inflammatory responses, potentially contributing to the progression of MIE to AO (Khatami, 2014; Thompson et al., 2015; Villeneuve et al., 2018). Severe and prolonged inflammation can become self-sustaining, and generate toxic products like reactive oxygen species (ROS) and cathepsins (released

during rupture of lysosomes), potentially causing extended tissue damage and increasing the risk or accelerating the onset of disease (Cox et al., 2020). Inflammation has been associated with target organ toxicities, such as heart, pancreas, liver, brain, lung, kidney, intestinal tract, and reproductive system and is emerging as an important node within the AOP network (He et al., 2011; Villeneuve et al., 2018). However, the complexity of inflammation, due to its multi-stage process poses a challenge in determining the KEs that capture the critical and measurable characteristics of inflammation (Villeneuve et al., 2018). The initiation of the inflammatory response is triggered by molecular stimuli falling into two main categories: pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (Šibíková et al., 2018). Specific pattern recognition receptors (PRRs), including transmembrane receptors (such as Toll-like receptors and C-type lectin receptors), as well as cytoplasmic receptors (such as RIG I-like receptors and NOD-like receptors), recognize PAMPs or DAMPs (Abbas et al., 2012; Šibíková et al., 2018). These PRRs are expressed across various cells, and their activation by PAMPs or DAMPs leads to the initiation of signaling pathways, including nuclear factor-κB (NF-κB) and MAP kinases, which result in the up-regulation of genes involved in inflammatory responses (Salvador et al., 2016; Šibíková et al., 2018). Exposure to both infectious and non-infectious stressors and cell damage can activate inflammatory cells and trigger inflammatory signaling pathways, including the NF-κB, and MAPK (Chen et al.,2018). Cells actively participating in the inflammatory response release microvesicles (MVs), and distinguishing and quantifying these MVs may contribute to determining the severity and progression of infectious and non-infectious inflammatory processes (Burger et al., 2013).

Organism response / health consequences (AO)

Clinically significant immunotoxic effects can be broadly classified into four main types including immunosuppression, immunostimulation, hypersensitivity and autoimmunity (Germolec, 2010; Semwal et al., 2022). Immunosuppression and immunostimulation manifest as the inhibition or (undesirable) overactivation, respectively, of immune cells or their functions in the immune tissues. These responses are collectively termed as “direct immunotoxic effects” (Wang et al., 2023). Immunosuppression can arise from the depletion of immune effector cells or the disruption of intracellular pathways essential for antigen recognition and other elements of the immune response (Rice, 2019). Various environmental contaminants have been shown to significantly suppress or enhance immune responsiveness depending on exposure level, time and route of exposure (Kreitinger et al., 2016). In certain scenarios, inflammation acts as a trigger for immunosuppression to prevent excessive immune activation and tissue damage (Zhao et al., 2015). Conversely, immunosuppression can lead to inflammation under specific circumstances, forming a feedback mechanism to control inflammation and maintain immune homeostasis (Bawanker et al., 2018). A compromised or suppressed immune system may render the body more susceptible to infections or inflammatory responses. For example, persistent immunosuppression has been shown to present a risk of cancer (Rice, 2019). For example, mercury has been identified as a potential epigenetic carcinogen, disrupting the intercellular communication of gap junctions (intercellular channels) and cause immunosuppression (Zefferino et al., 2017). Furthermore, cadmium has been shown to cause immunosuppression, along with immunostimulation (Sures & Knopf, 2004).

While immunostimulation can be beneficial to organisms to some extent, excessive or inappropriate stimulation may interfere with normal defense mechanisms, prompting responses that are ultimately harmful to the host (Wang et al., 2023). In some cases, immunostimulation can lead to hypersensitivity and/or autoimmune reactions, both characterised as “indirect immunotoxic responses”(Wang et al., 2023). Hypersensitivity reactions result from tissue damage or dysfunction following repeated stimulation by the same allergen, and often involves the antigen–antibody reaction. Autoimmunity is a process where the immune system targets its own components due to dysfunction and regulatory disorder (Sobel et al., 2005; Wang et al., 2023). For example, exposure to azoxystrobin and iprodione fungicides has been shown to induce immunotoxic effects, leading either to hyperactivation of immune system or to an alteration of the immune system that trigger immunosuppression (Naasri et al., 2020). In addition, environmental pollutants such as 3,3',4,4'-tetrachlorobiphenyl have been shown to cause thymic atrophy (i.e., shrinking of thymus) consequently resulting in immunosuppression (Lai et al., 1994).

Table 3. *Some of the well-known cytokines and their respective functions (source: Chen et al., 2018). Hundreds of other cytokines have been identified, each with critical roles in immune regulation.*

Cytokine	Family	Main sources	Function
IL-1β	IL-1	Macrophages, monocytes	Pro-inflammation, proliferation, apoptosis, differentiation
IL-4	IL-4	Th-cells	Anti-inflammation, T-cell and B-cell proliferation, B-cell differentiation
IL-6	IL-6	Macrophages, T-cells, adipocyte	Pro-inflammation, differentiation, cytokine production
IL-8	CXC	Macrophages, epithelial cells, endothelial cells	Pro-inflammation, chemotaxis, angiogenesis
IL-10	IL-10	Monocytes, T-cells, B-cells	Anti-inflammation, inhibition of the pro-inflammatory cytokines
IL-11	IL-6	Fibroblasts, neurons, epithelial cells	Anti-inflammation, differentiation, induces acute phase protein
IL-12	IL-12	Dendritic cells, macrophages, neutrophils	Pro-inflammation, cell differentiation, activates NK cell
TNF-α	TNF	Macrophages, NK cells, CD4+lymphocytes, adipocyte	Pro-inflammation, cytokine production, cell proliferation, apoptosis, anti-infection
IFN-γ	INF	T-cells, NK cells, NKT cells	Pro-inflammation, innate, adaptive immunity, anti-viral
GM-CSF	IL-4	T-cells, macrophages, fibroblasts	Pro-inflammation, macrophage activation, increase neutrophil and monocyte function
TGF- β	TGF	Macrophages, T cells	Anti-inflammation, inhibition of pro-inflammatory cytokine production

II Appendix: Bioassays for evaluation of immune response

***Table 4**: Bioassays to evaluate different aspects of immune responses in response to chemical contamination in drinking water sources as well as food. (Adapted from: Descotes, 2014; Escher et al., 2021; Finlayson et al., 2022; Wang et al., 2023).*

Aspect	Bioassays	Biological system	Immune response	Sensitivity	Throughput
Immunosuppression	Myelotoxicity - *Colony Forming Units-Granulocyte/Macrophage (CFU-GM) Assay*	*In vitro*	Innate	High	Medium
	(Lymphotoxicity) *Viability assays* - 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), - lactate dehydrogenase (LDH), and - trypan blue dye exclusion - ***Functional assays*** - *Lymphocyte proliferation assay (LPA)* - *Whole blood cytokine release* - *Natural Killer (NK) cell* - *T-cell-dependent antibody response (TDAR)* - *Dendritic Cell (DC) Maturation* - *Mixed Leukocyte response (MLR)* - *Cytotoxic T lymphocyte (CTL)* - Antibody production		 Innate/ adaptive Innate Adaptive Adaptive Innate Adaptive	 High High High High High High	 Low-medium Medium Medium Low Low – medium Low - Medium
	Other assays - Mast cells/Basophils - Cytokine production assay - Wound healing and regeneration assay - Transcriptomic profiles and/or RNA sequencing for MAPK signaling - Oxidative stress and antioxidant response assay (*Nrf2 assay)*	*In vitro / In vivo*	Innate / Adaptive	 High High Medium High High	 Low-medium High Low -medium Low -medium High

Aspect	Bioassays	Biological system	Immune response	Sensitivity	Throughput
	- ROS production assay				
Immunostimulation	Cytokine production assays - *Multiplex Assays* - *Enzyme-linked Immunosorbent Assay (ELISA)* - *Enzyme-Linked ImmunoSpot (ELISPOT)* - *NF-κB Reporter Assay*	*In vitro/in vivo/ex vivo*	Humoral and cellular	 High High High High	 High Medium Low-medium Medium-High
	Natural killer (NK) cell assays	*In vitro/In vivo/ ex vivo*	Innate	High	High
	Dendritic cell maturation assay	*In vitro/ex vivo*	Cellular	High	Medium
	Antibody production assay - *T-cell dependent antibody response assay)*	*In vivo*	Humoral	High	Medium
Hypersensitivity	- Human Cell Line Activation Test (h-CLAT) - U-SENS™ assay: - IL-8 Luc test using THP-G8 cell line - Genomic Allergen Rapid Detection (GARD) IL-1α and IL-1β biomarker assay for skin sensitization Local lymph node assay (LLNA)	*In vitro* *In vitro* *In vitro* *In vitro* *In vitro* *In vivo*	Innate Innate Innate Innate Innate Cellular immune response	Medium Medium Medium High Medium High	High High High Medium Medium low
Autoimmunity	C-Reactive Protein (CRP) Delayed-type hypersensitivity (DTH) - T-dependent antibody response (TDAR)	*In vitro* *In vivo*	Humoral immune response Cellular immune response	High High	Medium Low

III Appendix: Substances identified with immune effects

Table 5. *List of the substances causing immune and other known effects, sourced from the IRIS, ECOTOX data base and other relevant references). The concentrations at which these substances exert immune effects are not included in the table as this was beyond the scope of this study.*

C= Carcinogenic; CV= cardiovascular; D= developmental; ED= endocrine disruption; G= Genotoxic; GI= gastrointestinal; Rsp; respiratory; Rn= renal; H= hepatotoxicity; Hm= hematological; N= neurotoxicant; R/D= reproductive/developmental effects; T = tumor induction; Th= Thyroid PPDB= ; Pesticide Property Database; p = possible (and/or no but no status identified); minus (-) sign= no (direct) evidence/sufficient data found

Note 1: The International Agency for Research on Cancer (IARC) has categorised PAHs in four groups, i.e., group 1 as carcinogenic to humans, group 2A as probably carcinogenic to humans, group 2B as possibly carcinogenic to humans, and group 3, as not classifiable as carcinogenic to human (Patel et al., 2020).

Chemical Category	Chemical Name	CAS No.	Immune effects	Other known effects	Relevant references
Plant protection product (PPP)	Carbendazim	10605-21-7	Yes	C^{p}, R/D, ED	*PPDB , Jiang et al. 2015*
	Glyphosate	1071-83-6	Yes	C^{p}, R/D^{p}, ED^{p}	*PPDB, Bai et al. 2022*
	Simazine	122-34-9	Yes	C^{p}, R/D^{p}, ED	*PPDB, Li et al., 2017*
	Imidacloprid	138261-41-3	Yes	R/D; N^{p}	*PPDB, Mazloum et al, 2023*
	Flonicamid	158062-67-0	Yes	C^{p}, R/D^{p}	*PPDB, USEPA, 2020, Liu et al., 2022*
	Metolachlor	51218-45-2	Yes	C^{p}, R/D^{p}, ED	*PPDB, Hartnett et al., 2012*
	Paclobutrazol	76738-62-0	Yes	R/D^{p},	*PPDB, USEPA, 2015, Kamble et al.,2019*
Pharmaceuticals	Diclofenac	15307-86-5	Yes	H, Hm, CV, GI, Rn	*Gomaa et al.,2017; NCBI, 2024a*
	Carbamazepine	298-46-4	Yes	CV, H, N, Rn,D	*Nolte et al 2023; NCBI 2024b*
	Bezafibrate	41859-67-0	Yes	-	-
	17beta-estradiol	50-28-2	Yes	-	-
Polycyclic Aromatic Hydrocarbons (PAHs)	Anthracene	120-12-7	Yes	Note [1]	*NCBI, 2024d*
	Pyrene	129-00-0	Yes	Note [1]	*NCBI, 2024e*
	Benzo(a)pyrene	50-32-8	Yes	Note [1]	*Li et al., 2019; NCBI, 2024f*
	Fluoranthene	206-44-0	Yes	Note [1]	*NCBI,2024g;*
	Phenanthrene	85-01-8	Yes	Note [1]	*NCBI, 2024h*
	Naphthalene	91-20-3	Yes	C^{p}, N,	*PPDB*
Per- and Poly fluoroalkyl substances (PFAS)[4]	PFOS	1763-23-1	Yes	H, D, ED, T	*Fenton et al., 2021*
	PFHxA	307-24-4	Yes	-	-
	PFOA	335-67-1	Yes	H, D, ED, T	*Fenton et al., 2021*
	PFDA	335-76-2	Yes	-	*Fenton et al., 2021*
	PFHxS	355-46-4	Yes	H, D, ED, T	*Fenton et al., 2021*
	PFBA	375-22-4	Yes	-	*Fenton et al., 2021*
	PFBS	375-73-5	Yes	Th, Rn	*Christy et al., 2019*
	PFNA	375-95-1	Yes	H, D, ED, T	*Fenton et al., 2021*
Plasticizers	Di(2-ethylhexyl) phthalate (DEHP)	117-81-7	Yes	C^{p}, R/D, ED, N	*ATSDR 2022*
	Dibutyl phthalate (DBP)	84-74-2	Yes	R/D, ED	*Xu et al., 2015*

[4] Perfluorooctane sulfonic acid (PFOS), Perfluorohexanoic acid (PFHxA), Perfluorooctanoic acid (PFOA), Perfluorodecanoic acid (PFDA), perfluorohexane sulfonic acid (PFHxS), perfluorobutanoic acid (PFBA), perfluorobutane sulfonic acid (PFBS), perfluorononanoic acid (PFNA)

Chemical Category	Chemical Name	CAS No.	Immune effects	Other known effects	Relevant references
	Bisphenol A	80-05-7	Yes	CV, R/D, ED, N	*Chen et al., 2018; Naidoo et al., 2021*
Aromatic amines	Aniline	62-53-3	Yes	C^P, R/D, N	*USEPA, 2007*
	3,5-dimethylphenol	108-68-9	Yes	-	-
	3,4-dichloroaniline	95-76-1	Yes	Rn, R/D, ED	*Barnett et al., 1992; Boscolo et al., 2018*
	N,N-dimethylaniline	121-69-7	Yes	Hm	NCBI, 2024x
Volatile Organic Compounds (VOCs)	Trans-1,2-dichloroethylene	156-60-5	Yes	-	*NCBI, 2024i*
	1,1,2-trichloroethane	79-00-5	Yes	H, N, Rsp	*NCBI, 2024j*
	Trichloroethylene	79-01-6	Yes	C, H, R/D, N	*NCBI, 2024k*
	Acetone	67-64-1	Yes	Hm, R, Rn, Rsp	*NCBI, 2024l*
	Benzene	71-43-2	Yes	C, GI, Hm, N	*NCBI, 2024m*
Heavy metals	Lead	7439-92-1	Yes	C, Hm, Rn, R/D, CV, N	*NCBI 2024n*
	Beryllium	7440-41-7	Yes	C, Resp	*NCBI, 20240*
	Cadmium	7440-43-9	Yes	C, GI, Rn, Resp	*Mirkov 2024; NCBI, 2024p*
	Chromium	7440-47-3	Yes	C, GI, Hm, R, Rsp	NCBI, 2024q
	Copper	7440-50-8	Yes	H, GI	*Mitra et al., 2012; NCBI, 2024r*
	Uranium	7440-61-1	Yes	D, Resp, Rn, N	*NCBI, 2024s*
	Zinc	7440-66-6	Yes	Hm, GI, Rsp	*NCBI, 2024t*
	Selenium	7782-49-2	Yes	CV, GI, H, N, Rn, Resp	*NCBI, 2024u*
	Arsenic	7784-42-1	Yes	Hm	*NCBI, 2024u*
Others	Carbon dioxide	124-38-9	Yes	CV	*NCBI, 2024v*
	Silica	7631-86-9	Yes	C, Rn, Rsp	*NCBI, 2024w*

IV Appendix: ToxCast Assays related to immunotoxicity

ToxCast Assays were selected based on their "intended target family" (either immunoglobulin, cytokine or cytokine receptor) and on intended target family sub (NF-kappa B). The following assays in Table 6 are available in the ToxCast database for this selection. As extra information, the number of tests available is given. This does not exactly correspond to individual tested substances but gives an indication of which assays are more tested.

Table 6. ToxCast assays related to immunotoxicity

Assay	Number of tests available	Assay	Number of tests available
ATG_NF_kB_CIS_dn	171	BSK_KF3CT_IL1a_down	131
ATG_NF_kB_CIS_up	61	BSK_KF3CT_IL1a_up	11
BSK_3C_IL8_down	124	BSK_KF3CT_IP10_down	133
BSK_3C_IL8_up	9	BSK_KF3CT_IP10_up	12
BSK_3C_MCP1_down	141	BSK_KF3CT_MCP1_down	122
BSK_3C_MCP1_up	6	BSK_KF3CT_MCP1_up	15
BSK_3C_MIG_down	69	BSK_LPS_CD40_down	156
BSK_3C_TissueFactor_down	139	BSK_LPS_CD40_up	1
BSK_3C_TissueFactor_up	23	BSK_LPS_IL1a_down	111
BSK_3C_uPAR_down	158	BSK_LPS_IL1a_up	13
BSK_3C_uPAR_up	7	BSK_LPS_IL8_down	120
BSK_4H_Eotaxin3_down	176	BSK_LPS_IL8_up	16
BSK_4H_Eotaxin3_up	1	BSK_LPS_MCP1_down	135
BSK_4H_MCP1_down	145	BSK_LPS_MCP1_up	6
BSK_4H_MCP1_up	3	BSK_LPS_MCSF_down	156
BSK_4H_uPAR_down	128	BSK_LPS_MCSF_up	2
BSK_4H_uPAR_up	8	BSK_LPS_TissueFactor_down	92
BSK_BE3C_IL1a_down	109	BSK_LPS_TissueFactor_up	13
BSK_BE3C_IL1a_up	13	BSK_LPS_TNFa_down	125
BSK_BE3C_IP10_down	148	BSK_LPS_TNFa_up	50
BSK_BE3C_IP10_up	1	BSK_MyoF_IL8_down	16
BSK_BE3C_MIG_down	74	BSK_MyoF_IL8_up	18
BSK_BE3C_PAI1_down	100	BSK_MyoF_PAI1_down	75
BSK_BE3C_PAI1_up	3	BSK_MyoF_PAI1_up	5
BSK_BE3C_uPAR_down	74	BSK_SAg_CD38_down	164
BSK_BE3C_uPAR_up	29	BSK_SAg_CD38_up	8
BSK_BT_sIgG_down	103	BSK_SAg_CD40_down	163
BSK_BT_xIL17A_down	49	BSK_SAg_CD40_up	5
BSK_BT_xIL17F_down	62	BSK_SAg_CD69_down	153
BSK_BT_xIL2_down	51	BSK_SAg_CD69_up	3
BSK_BT_xIL6_down	54	BSK_SAg_IL8_down	121
BSK_BT_xIL6_up	9	BSK_SAg_IL8_up	24
BSK_BT_xTNFa_down	55	BSK_SAg_MCP1_down	139
BSK_CASM3C_IL6_down	126	BSK_SAg_MCP1_up	6
BSK_CASM3C_IL6_up	9	BSK_SAg_MIG_down	85
BSK_CASM3C_IL8_down	70	LTEA_HepaRG_FAS_dn	23

Assay	Number of tests available	Assay	Number of tests available
BSK_CASM3C_IL8_up	7	LTEA_HepaRG_FAS_up	34
BSK_CASM3C_MCP1_down	123	LTEA_HepaRG_IL6_dn	23
BSK_CASM3C_MCP1_up	19	LTEA_HepaRG_IL6_up	39
BSK_CASM3C_MCSF_down	125	LTEA_HepaRG_IL6R_dn	92
BSK_CASM3C_MCSF_up	12	LTEA_HepaRG_IL6R_up	12
BSK_CASM3C_MIG_down	83	LTEA_HepaRG_NFKB1_dn	77
BSK_CASM3C_TissueFactor_down	102	LTEA_HepaRG_NFKB1_up	15
BSK_CASM3C_TissueFactor_up	12	LTEA_HepaRG_TNFRSF1A_dn	70
BSK_CASM3C_uPAR_down	134	LTEA_HepaRG_TNFRSF1A_up	8
BSK_CASM3C_uPAR_up	22	TOX21_NFkB_BLA_agonist_ratio	45
BSK_hDFCGF_IL8_down	76	TOX21_RXR_BLA_Agonist_ratio	307
BSK_hDFCGF_IP10_down	187		
BSK_hDFCGF_IP10_up	8		
BSK_hDFCGF_MCSF_down	155		
BSK_hDFCGF_MCSF_up	5		
BSK_hDFCGF_MIG_down	122		
BSK_hDFCGF_MIG_up	1		
BSK_hDFCGF_PAI1_down	159		
BSK_hDFCGF_PAI1_up	9		